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# ANATOMICAL AND RADIOLOGICAL PARTICULARITIES OF THE AUTOPODIUM AT THE DOMESTIC SOLIPEDES.

**ADRIANA ALISTAR, G. PREDOI, C. BELU, C. VLĂGIOIU**
Faculty of Veterinary Medicine Bucharest

**Key words:** radiological particularities, autopodium, horse, donkey..

**SUMMARY**

The carpal bones, at donkeys in generally, have the shape similar to those of the horses. The most important difference is the flattening of the articular surfaces of the proximal row. The capitates connection with the second metacarpus is extremely low or even absent. The main metacarpus at donkeys is narrower and less flattened than at the horse. The corresponding surface of the capitate bone is more flat. The rudimentary metacarpal bones are relatively longer than at the horse. The first phalanx is narrower, appearing longer than at the horse. There is also a reducing of the depth to the median groove on the proximal end. The small sesamoid is shorter and thicker, and the third phalanx is transversely narrower comparative with the one at the horse.

The main elements of the anterior member, the stylopode and the zeugopod, are relatively conservative, maintaining their primitive morphology in most of evolutionary history of tetrapodes (Barone, 1966; Mişcalencu, 1982).

On the other side, the autopodium which forms the distal extremity of the member became very specialized, especially at mammals (Gheţie şi Hillebrand, 1971). Perissodactylele ongulate, which include solipedes, have only one evolved metapode and the extremity of the acropode covered in a hoof. These two regions are strong enough, relieved by muscular masses, which permits these animals rapid movement (et al Sisson 1975).

However, most affections of the locomotor system affect this acropodial zone, so the details of its morphology must be known.

## 1. MATERIALS AND METHODS

The study material was represented by ten horse toracal autopodes and ten donkey toracal autopodes. Animals destined for dissection, demonstration and reasearch in the Domestic Animal Anatomy Laboratory from The Faculty of Veterinary Medicine were used. After the achievement of dorso-palmar and latero-lateral radiographs, for the bone study, the autopodes were macerated in differente pots and labeled.

## 2. RESULTS AND DISCUSSIONS

Considered as a whole, both in anatomical and radiological terms, there can be distingueshed six sides of the carpal region.

The anterior side is separate by the forearm bones and the metacarp by sinuous lines. Slightly convex from one side to the other, flat from top to bottom, she dose not show any notable relief. The excavations of the adjoining side of the schapoid and the semilunar bone define an scapho-lunar interosseous duct (Fig.1).

The external side, formed by the pyramidal and the unciform bone, extends back and proximal with the pisiform bone. In its passage, the lateral collateral ligament fixes on the tubercle of the first two bones.

The back or the palmar side is irregular . It presents tuberous reliefs of the scaphoid, the semilunar and the unciform bone. A spacious fossa exists defined proximally by the first two bones and distal by the capitate and the trapezoid bone. On fresh parts these irregularities are leveled by an fibrous expansion, the common posterior ligament of the carpal bone.

The internal side is formed dorsal by the scaphoid and ventral by the unciform and capitate. It is almost flat and it widens to the base. The upper side articulates and it includes a glenoid cavity formed by the piramidal and the pisiform bone, an transversal oblonged condyle on the anterior side of the semilunar and the scaphoid bone and two cavities in the bach of this condyle.

The lower side is also an articular bone, relatively flat and it overlaps in the corresponding extremities of the metacarpal bones. At donkeys the proximal and distal articular surfaces of the first row of carpal bones are more flattened compared to the ones from the horse (Fig.2).

The main metacarpal bone presents on the palmar side surfaces of rough sinartrosis, on which the intermetacarpal ligament inserts itself. The articular carpal surface is represented by a wide range, designed for the capitate bone, and a narrower one, lateral, designed for the unciform bone.

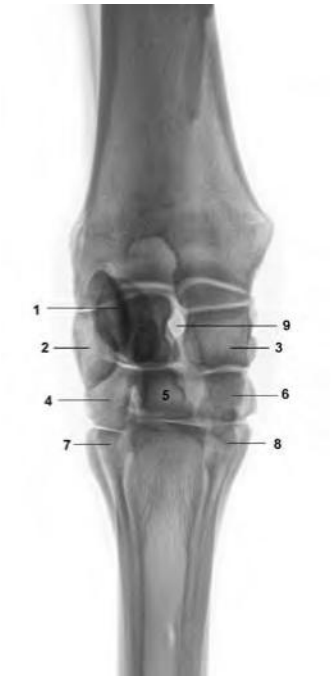


**Fig. 1 Radiography of the carpal region, right leg, horse, dorsal aspect**

**1-the pisiform bone overlaps the radiological image of the semilunar bone, 2-pyramidal bone; 3-semilunar bone; 4-unciform bone; 5-capitat bone; 6-trapezoid bone; 7-the proximal extremity of the IVth rudimentary metacarpal bone; 5- the proximal extremity of the IInd rudimenrary metacarpal bone**

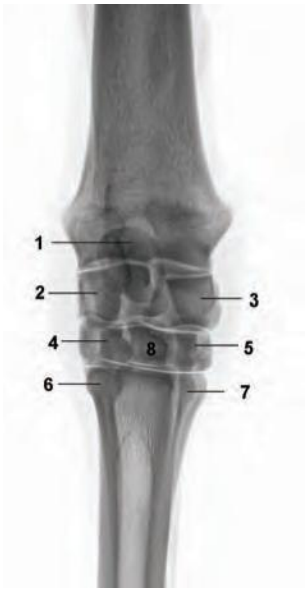


**Fig. 2 Radiography of the carpal region, right leg, donkey, dorsal aspect**

**1-the pisiform bone overlaps the radiological image of the semilunar bone, 2-pyramidal bone; 3-semilunar bone; 4-unciform bone; 5-trapezoid bone; 6- the proximal extremity of the IVth rudimentary metacarpal bone; 7-the proximal extremity of the IInd rudimenrary metacarpal bone; 8-capitat bone**

On the caudal outline of the extremity and on each side two diartroidial surfaces can be identified, flat, reduced, which correspond to the proper metacarpal bones. At the distal extremity, the inner condyle is slightly bigger than the outer one.

The inner small metacarpal bone is always thicker and longer. The carpal articular surface in formed by two facets which corespond to the capitat and the trapezoid. The head of the inner small metacarpal bone has a single facet, designed for the unciform.

The lateral metacarpal bones are relatively longer at the donkey. The upper articular surfaces tend to reach the same level as the large metacarpal bone.

The first phalanx at the donkey is narrower and the depth of the median ditch from the upper extremity is reduced (Fig.3,4). The second phalanx is longer than wide at donkeys and the relief, which separates the two glenoid cavities of the proximal extremity, is flattened. The third phalanx is narrower and the angles are less proeminent.

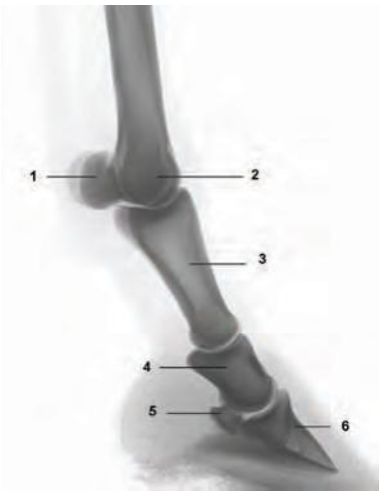


**Fig. 3 Radiography of the acropodial region, right leg, horse, sideways**

**1-sesamoid bones of the 1st digital joint; 2-the distal extremity of the large metacarpal bone; 3- first phalanx; 4-second phalanx; 5-sesamoid bones of the 3rd digital joint; 6-third phalanx**

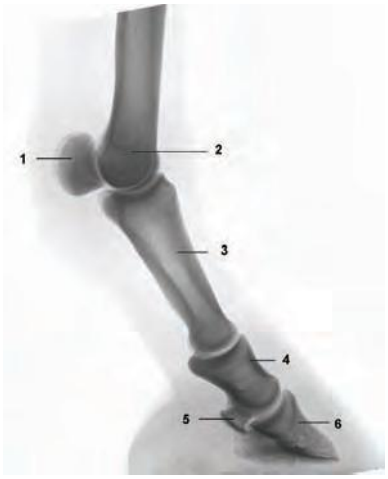


**Fig. 4 Radiography of the acropodial region, right leg, donkey, sideways**

**1- sesamoid bones of the 1st digital joint; 2-the distal extremity od the large metacarpal bone; 3-first phalanx;4-second phalanx; 5-sesamoid bones of the 3rd digital joint; 6-third phalanx**

## 3.CONCLUSIONS

3.1. In general, it can be concluded that in the radiological images of the carpal region it can be seen that at donkeys the articular surfaces, of the proximal row, are more flattened than at horses.

3.2. The capitats connections with the ext/int small metacarpal bone , at donkeys, is reduced or missing.

3.3. The large metacarpal bone, at donkeys, is thicker, less flettened craniocaudal.

3.4. The ext/int small metacarpal bones are relatively longer at donkeys. The superior articular surfaces tend to reach the same level with the large metacarpal bone.

3.5. The first and second phalanx are relatively longer at donkeys and the third phalanx has less proeminent angles.

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# LABOUR MARKET INTEGRATION OF VETERINARY MEDICAL STUDENTS – PRACTICAL TRAINING

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**Key words:** veterinary students, practical training, ITC skills

**SUMMARY**

Project aims to facilitate the transition to the work place of 2800 students through the use of innovative programs with practical training support, including information technology and communication resources; to develop and strengthen existing partnerships in order to facilitate students' labor market integration by involving at least 25 institutions and companies in trying to diversify the workplace experience (farms, clinics, laboratories etc).Project implementation will increase the relevance of the learning process and its outcomes by using innovative and interactive approaches for conducting 5 internship programs that cover the practical needs and allow the application of knowledge acquired in the classroom and also will update professional skills of the personnel involved in the practical training of students.

## PROJECT PARTNERSHIP

University of Agronomical Sciences and Veterinary Medicine Bucharest

University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad" Iasi

S.C. Softwin S.R.L

## PROJECT OBJECTIVES

- Facilitate the transition to the work place of 2800 students through the use of innovative programs with practical training support, including information technology and communication resources;
- Develop and strengthen existing partnerships in order to facilitate students' labor market integration by involving at least 25 institutions and companies in trying to diversify the workplace experience (farms, clinics, laboratories etc)
-Increase the relevance of the learning process and its outcomes by using innovative and interactive approaches for conducting 5 internship programs that cover the practical needs and allow the application of knowledge acquired in the classroom;

- Update professional skills of the personnel involved in the practical training of students.

## PROJECT ACTIVITIES

-Setting up a joint group of experts composed of representatives of beneficiary institutions and professional organizations that are coordinating the internships

-Develop and strengthen existing partnerships in order to facilitate students' labor market integration (that will be achieved by the Joint Group of Experts)

-Organizing the practical training.

-Design and development of innovative computer type digital collaboration platform support materials for practical training, for students, teachers, coordinators and tutors

-Assist staff internships partners to meet the responsibilities as tutors in the practical training of students.

-Organize awareness and information campaigns on the importance of practical training in the workplace so that graduating students will adapt to the demands of today's workplace.

-Implementation, monitoring and evaluation of practical training for students.

-Dissemination of project results.

## PROJECT TARGET GROUP

Staff from professional and beneficiary institutions as tutors – 25

Students participating in work placements (internships) – 2800

## PROJECT RESULTS

- Web Site Project
- IT access for the students
- 25 agreed and signed partnership agreements with clinics / companies / public institutions
- 25 certified tutors for practical training for students
- 2 modules developed as support materials related to practical training on skill levels (preclinical and clinical skills)
- 5 programs of related practical training sessions for the five years of study

- 5 categories of tools for the monitoring and evaluation of teaching practice

- 2800 students participating in internships

- 5 partial assessment and 5 final assessments for the evaluation of results obtained by students in practical training with practical evidence / information system using the facilities

## EQUAL OPPROTUNITIES

***Project management*** - In the frame of the management team will ensure the project will draw on experience of individuals regardless of gender, age or status, distribution of tasks based on competence criteria

***The target group*** - The principle of equality was explicitly sought by the criterion of selecting the target group, applying non-discrimination between male and female program participants.

***Content of training*** - Special emphasis will be placed on practical work and application of educational content regarding "Promoting equal opportunities in veterinary medical education".

## ADDITIONAL IMPORTANT ELEMENTS OF THE PROJECT

- Increasing the number of veterinary students introduced in real life workplace.

- Introducing modern (updated) equipment in a rural vet clinic.

- Innovative computer system that will contain background materials, and increasing the level of preparedness, adaptability and rapid integration.

## PROJECT SUSTAINABILITY

- In the management of institutions involved - Project partnerships between university centers and institutions / companies will carry on after completion of the project to ensure continuity of employment and employability of generations of students.

- In regard to the initial training - The main result of the project is developing practical skills of students. The learning support, practical training and tutors trained by the project are key elements in the implementation of future projects on higher education in veterinary medical services.

- Use of ITC skills acquired in the project - Based on systems developed under the project we will build new projects, will be able to

add new practical applications so as to ensure a sustainable practical training.

**ACKNOWLEDGEMENT:**

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# INFLUENCE OF COPPER AND ZINC SUPPLEMENT ON HOCK LESIONS SCORE IN ROMANIAN BLACK PIE DAIRY COWS

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**Key words**: dairy cows, hock lesions, copper sulphate, zinc sulphate.

**SUMMARY**

A 20 weeks study was undertaken in a farm of Iasi county, Romania, on four equal groups, each group formed of ten Romanian Black Pie dairy cattle (copper, zinc, copper-zinc and control groups) 10 to 50 – day lactation, with the aim to evaluate the hock lesions score and to corelate these score with their zinc and copper status according with the administration of these trace elements in sulphates form (2 versus 8,5 ppm copper and 9 versus 42 ppm zinc). Out of 40 examined cattle, 22 (55%) had the hocks periarthritis from which 21 were multiparous and one primiparous (group Copper-Zinc). During the study, hock lesions score showed higher values in cattle from Control group compared with cattle that received mineral supplements, the differences being statistically significant ($P < 0.05$). The tarsal periarthritis did not significantly affect plasma copper and zinc. A high percentage of the hocks periarthritis, in a farm, signifies a stall too short, too narrow with insufficient bedding and slatted floors. Adding bedding several times per week may reduce the incidence of hock lesions.

Tarsal periarthritis represents the chronic cellulitis of the skin and subcutaneous tissue of the lateral side of the hock, forming an adventitious bursa with interconnected pockets. One or both hocks may be involved. Infection of the bursa followed by a chronic discharging tract or peritarsal abscess formation is a common complication (Amstel and Shearer, 2006). Adequate mineral nutrition may be used as a strategy to optimize immune system function by reduction of metabolic and oxidative stress. Zinc and copper supplementation has been associated with high antioxidant activity. The aim of this study was to evaluate the hock lesions score and correlate this score with their zinc and copper status.

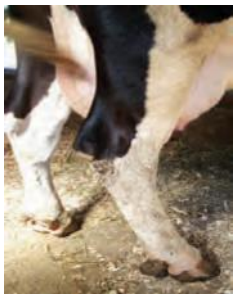
## 1. MATERIALS AND METHODS

A survey was conducted in a farm from Iasi county, Romania on 40 cattle divided in four equal groups, each group being formed of ten Romanian Black Pie dairy cattle (copper, zinc, copper-zinc and control groups) 10 to 50 day lactation. The cattle received 2 g

$CuSO_4$/cattle/week (Copper group), 2 g $CuSO_4$ and 10 g $ZnSO_4$/cattle/week (Copper-Zinc group) and 10 g ZnSO4/cattle/week (Zinc group) for 20 weeks period. These salts of Cu and Zn were dissolved in water before being orally administered in cattle. A detailed clinical examination was conducted in all 40 cattle, from the first day of the study, focusing on hock lesions score and individual hygiene score (ano-genital area, posterior udder area, posterior member - from above the hock till the studs, udder and belly area - in front of udder until navel) once a month. Blood was collected from the coccigiene vein in tubes with EDTA and heparin in order to evaluate the haematological profile, plasma copper and zinc.

## 2. RESULTS AND DISCUSSION

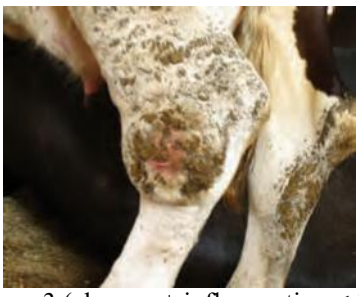
In A farm cattle received corn silage which is poor in all oligo-elements. Ratio analysis for dairy cattle, confirmed that the concentration of copper (2 ppm) and zinc (9 ppm) was insufficient. Cattle belonging to zinc and or/ copper supplemented groups had a daily intake of 8.5 ppm copper and 42 ppm zinc. In A farm, during the study, 5 cattle/40 were slaughtered, only 35 remained by the end of the protocol (one in Copper group, 2 in zinc group, one in Control group and one in Copper-Zinc group). The five cattle (12.5%) showed an increased weight loss, anemia and periarthritis at the hock. Hock lesions was a major disorder in this farm. The causes are varied: food deficiency (Cu, Zn, biotin, methionine, cysteine), stall, no adjustment program of hooves, infectious agents, genetics. Periarthritis of hock is a frequently observed disorders in cattle (fig. 1.) that have completed the protocol: 17 cattle/35 presented periarthritis at this level (6 from the Control group, 3 from Copper group, 5 from Copper-Zinc group and 3 from Zinc group). During the study, hock lesions score (table 1.) showed higher values in cattle from Control group compared with cattle that received mineral supplements, the differences being statistically significant ($P < 0.05$). From the 17 cattle, one was a primiparous (Copper-Zinc group).



Score 0 (normal hock)

Score 1 (alopecy)

Score 2 (alopecy + inflammation)



Score 3 (alopecy + inflammation < 10 $cm^2$)

Score 4 (alopecy + inflammation > 10 $cm^2$+pain)

**Fig. 1. Hocks lesions score in dairy cattle**

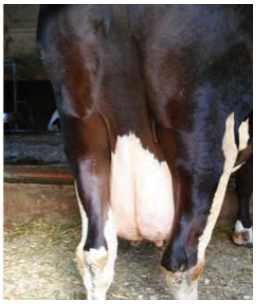
***Table 1.***

**Evolution of hock lesions score in the cattle from the 4 groups**

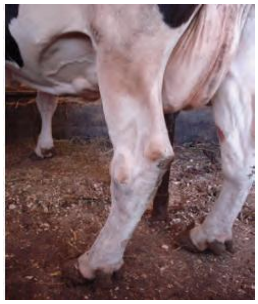
| Health Indicator | Time | Groups | | | |
|---|---|---|---|---|---|
| | | **Copper** | **Zinc** | **Copper-Zinc** | **Control** |
| **Hock lesions score** | **t0** | 0,90±0,56 n=10 | 0,50±0,52 n=10 | 0,50±0,70 n=10 | 0,60±0,69 n=10 |
| | **t1** | 1,20±0,78 n=10 | 1,40±1,34 n=10 | 0,77± 0,66 n=9 | 0,88±0,92 n=9 |
| | **t2** | 1,00±0,94 n=10 | 1,25±1,28 n=8 | 1,00±0,86 n=9 | 1,22±0,83 n=9 |
| | **t3** | 1,55±1,01 n=9 | 1,37±1,06 n=8 | 1,22±0,44 n=9 | 1,55±1,01 n=9 |
| | **t4** | 1,55±1,01 n=9 | 1,37±0,74 n=8 | 1,22±0,44 n=9 | 1,77±0,66 n=9 |
| | **t5** | 1,44±0,72 n=9 | 1,5±0,75 n=8 | 1,44±0,52 n=9 | 1,77±0,66 n=9 |

**n= cattle number; t0= first study day; t1= 28th study day; t2= 56th study day; t3= 84th study day; t4= 112th study day; t5= 140th study day**

The high percentage of hocks periarthritis signifies a stall too short, too narrow and slatted floors with insufficient bedding (Amstel and Shearer, 2006). Primiparous cattle, which are smaller than multiparous cattle were better adapted to the stalls system. Despite the fact that the hocks lesions score worsened significantly (P <0.05) in cattle from Control groups, from the beginning till the end of study, there was no significant impact on plasma copper and zinc. Since t3, the amount of litter for each cattle per day was doubled, reaching from 1 to 2 kg sawdust (fig.2.).



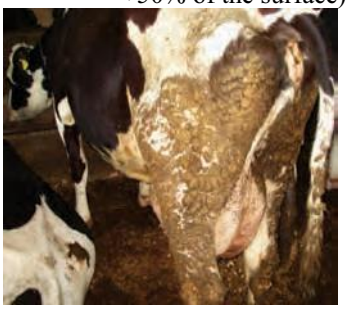
Note 0 („very clean")



Note 0,5 (some little dirt expanses)

Note 1 (dirt expanses on < 50% of the surface)

Note 1,5 (dirt expanses on > 50% of the surface, but no thick crust)



Note 2 (completely dirty area/ or covered with a thick crust)

**Fig. 2. Individual hygiene in the cattle from 4 groups**

Following this change, individual hygiene score improved significantly (P <0.01) in Zinc (at t5), Copper (at t5), Copper-Zinc (from t4) and Control (at t5 ) groups (table 2.).

***Table 2.***

**Evolution of individual hygiene score in the cattle from the 4 groups**

| Health Indicator | Time | Groups | | | |
|---|---|---|---|---|---|
| | | Copper | Zinc | Copper-Zinc | Control |
| **Individual** | **t0** | 3,05±1,69 n=10 | 2,90±1,22 n=10 | 2,80±1,13 n=10 | 3,20±0,71 n=10 |

| | | | | | |
|---|---|---|---|---|---|
| **hygiene score** | **t1** | 2,70±1,05 n=10 | 2,85±0,85 n=10 | 2,33± 0,70 n=9 | 2,77±0,71 n=9 |
| | **t2** | 2,95±1,36 n=10 | 3,75±1,94 n=8 | 3,00±1,06 n=9 | 3,83±1,17 n=9 |
| | **t3** | 3,44±1,42 n=9 | 2,87±1,24 n=8 | 2,66±0,90 n=9 | 3,61±1,13 n=9 |
| | **t4** | 1,77±0,97 n=9 | 3,37±1,09 n=8 | **1,61±0,96* =9** | 1,94±1,10 n=9 |
| | **t5** | **1,55±0,39* n=9** | **1,87±0,51* n=8** | **1,72±0,61* n=9** | **1,72±0,44* n=9** |

n= cattle number; t0= first day of study; t1= 28th day of study; t2= 56th day of study; t3= 84th day of study; t4= 112th day of study; t5= 140th day of study;
* Significant differences (P<0.01) in the group reported at t3

Hematology profile revealed that the mean of erythrocytes total number (RBC) in cattle from Copper-Zinc group was significantly higher (P<0.05) at t5 (6.37 ± 0.46 x $10^6$/mm3) compared to t0 (5, 63 ± 0.53 x $10^6$/mm3). Normal values of RBC in cattle are 5.00 to 10.00 x $10^6$/mm3 (Radostits et al., 2007). During the entire study, no significant differences were noted in cattle receiving mineral supplement in comparison with cattle from Control group. Hemoglobin and hematocrit of minerals supplemented cattle were not significantly different than cattle of the Control group. Cattle from the control group had increased (11.37 ± 3.21 x $10^3$/μL) mean of white blood cells (WBC) in comparison with the supplemented groups, but the differences were not statistically significant (P>0.05). Means of platelets of cattle from Control group were higher compared with the supplemented groups, but differences were not statistically significant (P> 0.05). The results of mononuclear leukocytes were not significantly different from supplemented groups compared to Control group. The increased amounts of lymphocytes occurred in cattle from Control group at t3 (7.1 ± 1.02 x $10^3$/μL), but the differences were not statistically significant compared with t0, t1, t2, t4 and t5. Regarding the evolution of polymorphonuclear leukocytes, there were no statistically significant differences (P> 0.05) between supplemented groups and Control group. Mean of neutrophils of cattle receiving mineral supplements were lower at t0 compared to other determinations, but the differences were not statistically significant (P>0.05), maintaining themselves in the physiological parameters (0.6-4, 12 x $10^3$/μL (Kramer, 2000). Cattle from Control group showed at t0 higher values of eosinophils (2.3 ± 0.11 x $10^3$/μL) statistically significant (P> 0.05) than t4 ( 1.2 ± 0.01 x $10^3$/μL) and t5 (0.9 ± 0.05 x $10^3$/μL). The results of eosinophils values from other groups of cattle are very similar among them.

Many factors may influence blood trace elements concentrations. For example, inflammation, stress (Herdt et al., 2000) and infection (Orr et al., 1990) may increase plasma copper and decrease plasma zinc. False „highs" for plasma copper and hypozincaemia during acute infections may be ruled out by discarding results for samples with high Cu : Zn ratios (>3–4) (Andrews et al., 2004). In our study the ratios of copper and zinc ranged between 0.67 to 1.25, well below 3-4.

During the 140 days study period was noted a significant evolution of plasma copper in each group, but since the second month of ratio supplementation, the cattle got normal limits (0.95 to 1.57 mg/L) (Radostits et al., 2007) of plasma copper. Since t4, cattle of Copper group (1.31 ± 0.16 mg/L) had statistically significant higher ($P<0.05$) values of cupremie compared with cattle of Control (0.92 ± 0.31 mg/L) ($P<0.01$) and Zinc (1.04 ± 0.14 mg/L) groups.

Starting with t1, significant differences were found ($P <0.01$) in cattle from two groups that received 10 g ZnSO4/week (Zinc and Copper-Zinc groups), compared with Control group. During the entire study, was noted a substantial change in plasma Zn in each group ($P <0.01$). Cattle supplemented with $ZnSO_4$ have reached the normal parameters of plasma Zn (0.8 to 1.2 mg / L) (Suttle, 2004), one month after administration of ZnSO4, while other groups, two months later. In our study, statistical Pearson test showed no correlation between inflammatory disease and plasma copper and zinc in cattle from the 4 groups.

## 3. CONCLUSIONS

3.1. During the study, hock lesions score showed higher values in cattle from Control group compared with cattle that received mineral supplements, the differences being statistically significant ($P <0.05$).

3.2. Pearson statistical test showed no correlation between plasma copper and zinc and hock lesions.

3.3. Adding bedding several times per week may reduce the incidence of hock lesions.

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# BIOECONOMIC AND ECO-ECONOMIC BASED IN THE PROGRESS OF VETERINARY MEDICNIE REGARDING REPRODUCTIVE BIOTEHNOLOGY FOR SUSTANABLE DEVELOPMENT OF MANKIND IN THE TWENTY-FIRST CENTURY

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## INTRODUCTION

The relations between humans and the animals they use as food have changed in time. At first, man was a hunter. Now hunting is a sport. Afterwards, he became a preserver, keeping only the wounded or young animals for food. Now keeping is a matter for the Zoo gardens. Later on, humans started to reproduce animals and became breeders. Now they are breeders of many animal species. Today some breeders, especially as far as poultry and pigs are concerned, have become producers. Now people speak about poultry and pig industries. This development has been determined by the increase in the human population in one part of the globe or another. Over the last century a human demographic explosion has taken place. So, the requirements for food, for commodities and for other goods has increased tremendously. Industries have grown very much and along the way so has the needs for transports and energy. The frames and trends of economy have to be changed. Agrifood must solve the problems of food security and of the safety food, in point of the quantity and the balance of nutrients needed by the humans. There are less fossil fuels and they have became very expensive. They should be substituted by bio gas and bio fuel or by non conventional sources of energy (such as the sun, the wind and the waves). It is obvious that all the organic synthesis solutions are connected to the prefixes “bio”, „eco”, „natural”, this rightly entitleing them to the name of “Green power”. This is the effect of the chlorophyll (the pigment of green plants, all the way from mere algae up to the big trees) capability to deposit sun energy into molecules of organic substances synthesized from CO2 and H2O adding in some cases N, P or even other chemical

elements. The Green power is amplified by biodiversity. It is obvious that a sustainable economy of the future has to become a bio-economy, adapted to the rural area and based on a large biodiversity that will create first of all an opportunity for more producers of primary organic synthesis and further on for a longer line of consumers up to the final state of dead organic matter that must be mineralized. In this context, Nicolas Georgescu-Roeger's world-wide-known Bioeconomics paradigm of improving the agricultural efficiency becomes most topical, particularly as mankind's limited natural resources are being depleted. These aspects have been emphasized even more by the current global economic and financial crisis. On the basis of the more recent Green Power paradigm, the paper also presents a new point of view about a possible new paradigm of sustainable rural bioeconomics. Nowadays a sustainable economy must become rural, based on Agrifood Biodiversity.

## INFORMATION SOURCES AND DISSCUSIONS

According to the data registered in the Romanian statistical yearbook for 2002, when the latest animal count was taken in Romania, the animal production development in EU member countries, estimated by the number of cattle and pigs and by the milk production per inhabitant, stood as presented in table 1. Data in the table show remarkable differences in the animal production levels of the EU countries.

As far as pork production is concerned, the index of head/inhabitant places Denmark in forefront with an index which is 2½ times higher than the second in line, Holland. But Holland and Belgium have high indexes as well. These threes are breeders'countries. Denmark created the best maternal breed, the Landrace, Belgium has created the meaty Pietrain breed and Holland is now offering the Seghers hybrid. Normal consumers are all other countries in the table providing between ¼ and ½ of pork per inhabitant. Romania used to be producers', as well, in 2002. Unfortunately, Romania is currently producing less pork for the market, but still enough hogs for the traditional Christmas pig treat.

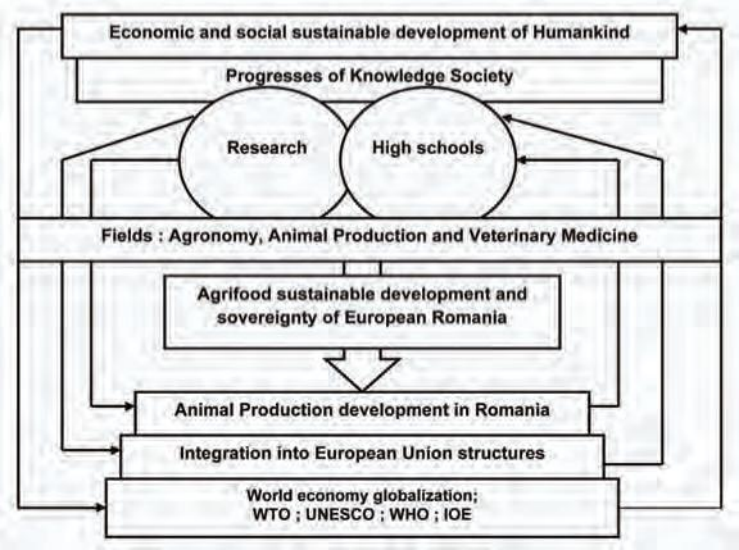
The low value registered in the UK and the fact that for Ireland this index in not mentioned is explained by the peculiar climate of the British Islands which is more convenient for pastures than for grain crops. Thus, the British choose, by tradition, beef or mutton over pork. Now, they act to implement the outdoor technology. Much complex is the question of cattle. One dual purpose cow of the European type, let's say a Fleckvieh

(Simmental), produces about 4200 kg of milk per year for the market. Also, she gives birth to 0.8 calves able to grow with a mean daily gain of 0.8 kg. That means the calf will get about 260 kg body weight per year. For a meat ratio of 48%, this leads to about 125 kg of meat. Whereas a man consumes 25 kilos of beef per year in addition to other kind of meat, one cow will cover the needs of 5 people. At the same time the cow will meet the milk needs of 20 people that consume 210 kg of milk per year. This imbalance in efficiency was corrected by the cattle breeders in USA who specialized cattle in dairy breeds, where cows are milked, and beef breeds, wher cow milk is sucked by the calf. In this way the cattle efficiency went up very much and nowadays many countries consider specialization of the cattle breeds. The British countries have created the beef cattle breeds and are the only European countries that have solved the question of specialization of cattle breeds. Belgium and France appear to be countries that take to the specialization of their cattle breeds. That is a target for all the EU member countries, including Romania. Sheep breeding presents little interest to the EU countries. Great Britain breeds sheep for mouton. So do many other countries. In Germany breeding sheep is viewed like a hobby. Only Spain, after joining the EU, increased its sheep stock. The explanation is simple for a market economy: the offer of sheep products on the EU free market was small. The same reason caused Italy to implement buffalo breeding, creating a new breed, the Mediterranean Buffalo. If cow milk is produced so much that it requires market protection by milk cottas and meat production is provided at a satisfactory level by poultry and pig industries, interest for other animal products or other farm animal breeds still exists. Biodiversity of farm animals makes the world richer with new species of birds and specialized new breeds of beef or dairy cattle. New breeds or hybrids are created in poultry or in pigs. Goat breeding is promoted as well and new technologies are in view for sheep production. All these trends act for more animal production in EU countries.

Animal production in Romania has taken a drastic fall over the last 20 years. Transition from the planned economy to the market economy was very costly. It is time to stop the decline and act for the regeneration of animal production. Statistics point to the dominance of ruminants in the livestock structure. There can be noticed a light decrease in the cattle livestock and an obvious increase in the sheep and goat livestock so that in 2007 there were more sheep and goats than cattle. May be this could be so due to the fact that sheep and goat products, especially meat, find it easier to penetrate the market .Most of the meat is pork, followed by poultry. Ruminants are producing together less meat than pork even if

these species have much more livestock expressed in EU. That means young ruminants are slaughtered early in their life. It is true that Romanians have the sacrificial Easter Lamb tradition, but as far as calves are concerned there isn't any sacrificial tradition. As far as milk is concerned, it is important to mention the case of the buffalo cow milk that can be produced in Romania over the EU quotas.

From the above facts one must conclude that Romania's animal production is way too low compared against the other EU countries and in relation to its own natural resources. This situation is mainly a result of the widespread subsistence agriculture. This feature of the Romanians accounts for the low efficiency of farm ruminants in meat production. Pork and poultry industries pertain mostly at private commercial companies and might register a sooner progress. For the domestic Romanian market of animal production of prioritary importance is the transition from the subsistence agriculture to a market agriculture, urging the concentration of land property in farms with a desired land surface of 25 – 30 ha. This type of family farms has to breed especially dairy cows and sheep. For this type of farms it is necessary to ensure special technical services such as artificial insemination, embryo transfer services, fed lots, wool shearing points, processing units or retailer shops for products and other. Small- and medium-sized cooperatives organized by farmers are the best status of such organizations. This is not an easy target since small land owners are tend to be hostile to the production cooperatives or associations. That issue should become the central policy of the Ministry of agriculture.



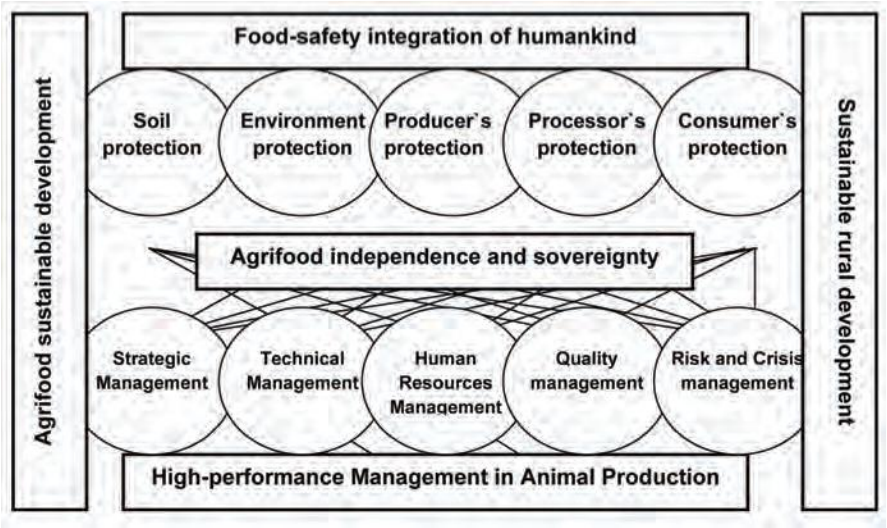



**Fig .1 The complex nature of the principles, targets and consequences for the concepts of agrifood independence and sovereignty, based on More animal production (orig.)**

Pork and Poultry industries could prosper with financial assistance and good advisory services, but cattle breeding and sheep breeding require changing the mindset of many people. The first able to penetrate the EU market are the buffalo and sheep products. Also of interest are the beef goods of commercial quality, lean sheep carcasses, lambs weighing 30-35 kilos and all the branded traditional products. The quantitative target for the domestic market is to attain a mean production level per inhabitant as reached by the 25 EU member countries (except for Romania and Bulgaria). Romania has the necessary human and material resources to hit this target.

***Tab.1***

**The Resulted "MORE ANIMAL PRODUCTION" potential of Romanian agriculture expressed by livestock and meat production of the 2025 year (Calculate original A.T.B. et all)**

| Romania Model - dynamics for the 2025 year | Resulted meat production potential | | Resulted livestock Potential | | Animals / inhabitants |
|---|---|---|---|---|---|
| | Meat live animals | Meat in carcasses | Total of livestock | Animals to be slaughtered | |
| | (thousand tones) | | (thousand heads) | | (no.) |
| | "MORE ANIMAL PRODUCTION" potential of Romanian agriculture expressed by BOVINE dynamics | | | | |
| | 726 | 385 | 6.740 | 1.967 | 0,30 |

| | | | | | |
|---|---|---|---|---|---|
| | "MORE ANIMAL PRODUCTION" potential of Romanian agriculture expressed by PIG dynamics | | | | |
| | 1.381 | 1.063 | 15.498 | 14.501 | 0.69 |

***Tab. 2***

**The Credibility of "MORE ANIMAL PRODUCTION" in Romanian agriculture expressed by the livestock and meat production counted as potential, compared to already existing models in EU countries (Calculate original A.T.B. et all)**

| EU countries referred to when MORE ANIMAL PRODUCTION in Romania was counted | | Animals / inhabitant | Potential livestock of Romania | | Potential meat production of Romania | |
|---|---|---|---|---|---|---|
| | | (no.) | (thousand heads) | | (thousand tones) | |
| "MORE ANIMAL PRODUCTION" potential of Romanian agriculture expressed by BOVINE dynamics | | | | | | |
| Already existing model | Ireland | 1,76 | 39.443 | 11.832 | 4.259 | 2.257 |
| | France | 0,34 | 7.619 | 2.285 | 822 | 435 |
| | Denmark | 0,34 | 7.619 | 2.285 | 822 | 435 |
| | Belgium | 0,34 | 7.171 | 2.151 | 774 | 410 |
| "MORE ANIMAL PRODUCTION" potential of Romanian agriculture expressed by PIG dynamics | | | | | | |
| Already existing model | Denmark | 2,16 | 48.407 | 44.534 | 4.453 | 3.428 |
| | Holland | 0,82 | 18.377 | 16.906 | 1.690 | 1.301 |
| | Belgium | 0,72 | 16.135 | 14.844 | 1.484 | 1.142 |

Progress in breeding was possible from intimate knowledge of breeding phenomena. Animal reproduction has many biological, technological and economic, which directly affect the quantity and quality of livestock production.

Biotechnology of Reproduction provides an outstanding contribution by its theoretical and practical content to enhance learning and improve the quality of modern livestock herds, high yield and economic efficiency, Bogdan AT,( 2002). The quantity and especially the production of semen quality is a primary means of intensive biotechnological breeding. The fertility of a breeding is strictly related to semen quality. I cannot ignore the economic benefits of applying some of the methods to increase production of milk, eggs, wool, animal weight gain, reducing non-productive period, introducing a series of disease resistance..Most geneticists are optimistic thoughts already turning to the creation of cloned transgenic cattle herds, the milk which they find extremely abundant medicinal interest. Animal reproductive

technologies have the potentiality to develop a wide variety of new varieties on the one hand, and on the other hand can increase productivity in animal husbandry. Some have gone even biotech animal to human, which can lead to progress, but in many cases, however, appeared more and more ethical issues. Therefore access to certain such techniques should be limited so as not to trigger further changes difficult to overcome for the human species.

Worldwide breeding using biotechnology for the advancement and development of livestock production.

The main breeding biotechnologies that are currently used: artificial insemination and embryo transfer.

Artificial insemination is a breeding system that suppresses human sexual contact between female and male directly, allowing this division of sperm production and hence amplifies the number of offspring that can be obtained from a male.

Technical components of this technology are: the semen collection, processing and portioning dilution of the dose, its conservation (if possible by freezing) and inoculated to females in oestrus.

In terms of livestock artificial insemination application has the following advantages: the major objective is the practice of artificial insemination and genetic improvement of the working population through intensive and rational use of high-value livestock breeding male on a female actually huge compared to what can be included by using the respective breeding mating: estimating the livestock breeding is done by a complex of criteria on which grants partial class reliability; reduction in the number of bulls used for breeding, triggers the application of rigorous selection, as their genetic capability. To this end, both globally and in our country, the assessment method is bulls "Progeny-test (after progeny testing), was admitted to breeding only those who proved to be good breeders, thus ensuring genetic progress from one generation to another (better vertical); frozen semen offers international exchange of genetic values, access to global performance in the field, enabling companies specializing in the field with remarkable effects in several countries with advanced animal husbandry, including the stands in Germany, USA, Austria, France and others; . implications should also be noted beneficial in protecting and maintaining the health of staff, having direct contact with the female male, avoid the dissemination of diseases through the first act of copulation (brucellosis, vibriosis, Trichomoniasis, etc..); artificial insemination (AI) entails enhancing male fertility, which allows increasing the intensity and

accuracy of their selection and, ultimately, increasing pressure by the male in the work of genetic improvement.

Embryo transfer (ET) is the technology of breeding that pregnancy is obtained from the embryo recipient female foreign eggs or embryos to be transferred. Technical components of this biotechnology are: selection of embryo donor females, for their stimulating hormone poliovulation monotocic species and species politocic ovulation, fertilization of ova in oviducts obtained maternal, embryo collection formed 7-8 days after mating or AI before implementing them in the womb and their transfer to recipient females synchronized with the phase of cyclical uterine age at which embryos were collected (fresh or after preservation by freezing), GF Toba (2000). Embryo is a biotechnology that pair formation is controlled by man and is also known as IA

The MOET is pressed to have more ovulations, not only by hormonal stimulation of maturation of ovarian follicles to increase the number of follicles dehiscence but also to reduce the interval between samples (washing) the success of embryos from the same donor, Paraschivescu M.Th. ( 2008, 2010). The more daring research has led to the induction and stimulation obtained immediately after lyses poliovulation lutea formed in place of follicles from which eggs and embryos collected later. Initially it was considered that the ET in cattle after poliovulation and especially the one after multiple ovulations, the donor cows would increase the intensity of selection, as selection intensity increased IA male genitors. It was found that raising the fertility of cows but also through the practice of MOET, is much weaker than the increase in fertility bulls gained IA. ET is a superior breeding IA in case of transfer of populations from one area to another because it eliminates the need to increase the absorption cross-checking required when using AI. When the transfer of a population is by IA participation by one parent, the replacement of blood is absorption cross over 5-6 generations. The transfer of a population by using ET is lower and it is comparable to that provided by additional imports of requiring only a period of 24-30 months as necessary for embryo inoculation and increased until puberty and installation heifers' pregnancy. This time, however, cannot be considered a waste of time because the incubation of embryos in the uterus of females belonging to the farm is transferred, use colostrums from mothers who have formed antibodies to the living environment of the new born calves were obtained and formation of antibodies to themselves in advance for this environment will favor organisms transferred accommodate increasing their chance of

acclimatization. In other animal species practice AI and ET is less advanced than in cattle there are differences in this regard, and among other species.

We recomande for Romania's scientific literature in the areas of agriculture in general and animal production in particular to use the Bioeconomy and Eco-Economy paradigms, particularly since the historic truth points to the absolute priority of the internationally-acknowledged ideas coined by Nicholas Georgescu-Roegen, the US scientist of Romanian origin.,Nicholas Georgescu-Roegen uses a concept taken from thermodynamics, i.e. entropy, and demonstrates that it is the most economic of all laws of nature. The relation between the economic process and the entropy law is only an aspect of general fact, i.e., this law is the foundation of life economics at all levels and explains the irreversible loss of energy, of depletable and more and more scarce resources. In these writings one finds the essential elements of his new concept, *bioeconomics,* defined by the systematic links between *economic processes, the environment* in which they take place and *man* as a living creature represening a combination of rational and irrational behaviours as well as a generator and a beneficiary of products and services. Besides the above elements, the Roegenian concept includes the following: connections between economic proceses and dynamic processes, the predominantly aleatory character of these processes as well as the specific institutional component. All are defining elements of the Roegenian Bioeconomics and form the subject mater of the new discipline  Bioeconomics became more clearly defined and devloped quickly owing to the contribution of several research centres, universities, professional asociations in Europe (France, Switzerland, Italy, Germany, etc.), Japan, Latin America as well as to numerous national and international conferences, discussions and debates.

Consequently, starting from an economy based on consumption and an excessive use of natural resourcs, Eco-Economy  (Lester Brown) postulates that Terra's natural resources need to be used rationally, based on environmental protection. Consecutively, these concepts, ideas and paradigms in the economic literatures of recent decades and particularly in those related to Bioeconomy (Georgesgu Roegen) and Eco-Economy have taken to the forefront of all the economic policies and of the social strategies a new paradigm that is indispensable to life on earth: the sustainable development. Lester Brown, rightly considerered to be one of the founders of the basic concept of sustainable development, shows that they allow for a reconciliation between economy and ecology by a rational use of the planet's resources.

In conclusion starting from our results regarding the More Animal Production in Romanian Agriculture, linked with the basic paradigms on Bioeconomy and Eco-Economy scientifically applied to the rural economy, in the context of a sustainable rural development, the possibility exists to issue the following tentative terms for consideration in the future: a.Bioeconomic sustainable development of the rural areas; b. Eco-Economic sustainable development of the agrifood production; c. Eco-Bioeconomic sustainable development of the agrifood green power.

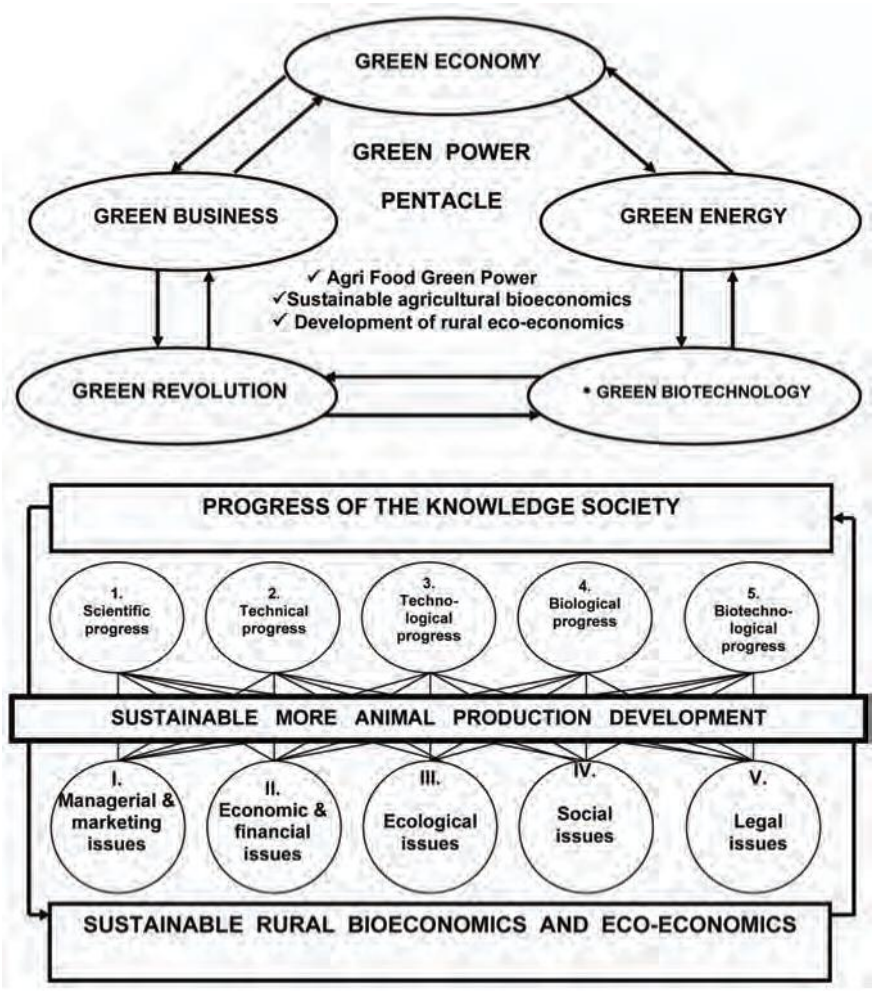



**Fig.2. The components of the Green Power pentacle, correlated with sustainable more animal production development based on sustainable rural bioeconomics and eco-economics (orig.)**

The More Animal Production must be a component of the Agrifood Green Power concept based on Green Economy, Green Business, (new)

Green Revolution and Green Energy – aspects underlying Lester Brown's more recent Eco-Economy – Building an Economy for the Earth concept, which we consider to be insipired after Nicholas Georgescu-Roegen's initial Bioeconomics concept. More about these issues can be found in the paper About Eco-Economy and the Green Economy in Romania by Bogdan A.T. and his team, recently printed by the Academy Publishing House. We consider the aspects related to the link between More Animal Production in Agriculture and the Agrifood Green Power to spell the need for Romania to work out urgently a medium- and long-term strategy of rural sustainable development.

Final conclusion: Starting from the title of the 1970 best-seller The Greening of America, and establishing a bridge in time, under the new circumstances of simultaneous and non-successive global crises the author's team believes the time is high for the Greening of Romania so that the country may become an Agrifood Green Power and play a major role in the contemporary world.

This paper is bibliographic material for postdoctoral school „ Livestock biodiversity and food biotechnology based on bioeconomy and eco-economy for ecosanogenesis"

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# TRANSMISSION OF *LISTERIA* SPECIES BY FOOD PRODUCTS FROM ANIMAL ORIGIN

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**Keywords:** *Listeria monocytogenes*, food, epidemiology, dairy

**SUMMARY**

The *Listeria* genus is constituted of six species: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimery, L. seeligeri*, and *L. grayi*. All of these species are found in the environment. The ubiquitous character of these germs results in the contamination of numerous food products. Epidemiological studies have demonstrated that *Listeria monocytogenes*, an opportunistic pathogen for human and animal organisms, is transmitted by food.
Different commercial food products (raw milk and dairy, vegetables, raw meat, poultry and fish), as well as fast food preparations are frequently contaminated by *Listeria* germs and prove to be the source of *Listeria* infection manifested by different clinical aspects of listeriosis (septicemia, meningitis, encephalitis, abortive disease), as result of the digestive transmission of these germs to humans.
Although diary products have been described as major source outbreaks of listeriosis, other raw or recontaminated products of animal or vegetable origin may serve as vehicles of transmission of this pathogen. *Listeria* spp. has been isolated from poultry, red meat and meat products, although these foods have not been associated with outbreaks of human listeriosis. *Listeria* spp. are capable of growing on both raw and coked meat at refrigeration temperatures. During the transformation processes from raw meat into meat products *Listeria monocytogenes* can be introduced, and the amount depends on the extent of contamination, personal and general hygienic measures, as well as the process parameters.

## 1. MATERIALS AND METHODS

A total number of 26 strains of *Listeria* spp. were investigated for identification/ confirmation. These strains were isolated from animal meet (sausages and other pork and beef preparations), poultry and diary products (Table 1).

In our study there were used:

- 5% defibrinated sheep blood agar;
- blood-free agar;
- Gram stain kit (crystal violet, stabilized iodine, decolorizer, safranine);
- 3‰ agar distributed in 16/160mm tubes;

- simple broth with 1% glucose, trehalose, D-mannitol, D-manose, L- rhamnose, D-xilose, and brohm-thymol blue as pH indicator;
- beta-haemolytic *Staphylococcus aureus* strain ATCC 25923;
- *Rhodococcus equi* strain ATCC 6939;
- hiperimmune rabbit adsorbed sera *Listeria monocytogenes* 1a and *Listeria monocytogenes* 4b.

***Table 1***

**Investigated strains (No./%)**

| Type of product | No. | % |
|---|---|---|
| Raw meat | 8 | 30.72 |
| Meat products | 15 | 57.69 |
| Diary products | 3 | 11.53 |

The bacterial identification included morphological (Gram staining, microscopic examination, motility, oblique illumination of colonies on blood-free agar) and biochemical (catalase, beta-hemolysis on 5% sheep blood agar, CAMP test, trehalose, mannitol, manose, rhamnose, xilose reactions) methods. *L. monocytogenes* strains were serotyped for serovar identification.

The following international recommended tests *(3, 6)* were performed:

1. Morphological aspects:
a) Gram staining and microscopic examination;
b) Beta haemolytic activity, on 5% sheep blood agar;
c) Motility, on 3‰ agar;
d) Oblique illumination of colonies, on blood-free agar.
2. Biochemical reactions:
a) Catalase production
b) Oxidase test
c) CAMP test
d) Acid from: glucose, trehalose, D-mannitol, D-manose, L-rhamnose, D-xilose.
3. Serological identification (agglutinating test with rabbit adsorbed antisera):
a) Antiserum *Listeria monocytogenes* 1a
b) Antiserum *Listeria monocytogenes* 4b.

## 2. RESULTS AND DISCUSSIONS

All of 26 strains examined were positive for *Listeria* spp. (Table 2). The investigations finally revealed:

- *7* strains (26.92%) *L. monocytogenes* (out of which 6 strains *L. monocytogenes* serotype 1a and 1 strain *L. monocytogenes* serotype 4b)
- 16 strains (61.53%) *L. innocua*
- 1 strains (3.84%) *L. gray*
- 2 strains (7.69%) *L. welshimery*.

Raw minced meat (Table 3) had the highest incidence of *Listeria spp.*, with 8 (30.72%) positive strains.

The incidence of *Listeria* spp. was found: 6 strains (23.07%) in fresh sausages (3 strains *L. monocytogenes* and 3 strains *L. innocua*), 5 strains (19.23%) in pork meat (3 strains *L. innocua* and 1 strain *L. monocytogenes*), and 1 strain (3.84%) *L. monocytogenes* in chicken meat.

*L. monocytogenes* strains were serotyped and they revealed:

- 6 strains *L. monocytogenes* 1a (serotype circulating in Romania);
- 1 strain *L. monocytogenes* 4b (serotype isolated from poultry carcass, imported from Italy)

*L. innocua* was identified in 16 strains (61.53%) among *Listeria* species. There were detected predominantly: 7 (26.92%) strains from raw minced meat, 3 (11.53%) strains from fresh sausages, and 2 (7.69%) from pork muscular tissue.

*L. welshimeri* was revealed in 2 strains (7.69%) from powder milk.

- 1 strain (3.84%) *L. gray* was detected in beef muscular tissue.

***Table 2***

**Identification of *Listeria* spp. strains (No. / %)**

| Tests \ Species | | *L.monocytogenes* | | | | *L. innocua* | | *L. gray* | | *L. welshi-meri* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | serotype 1a | | serotype 4b | | | | | | | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % |
| Beta-haemolysis | | 6 | 23.07 | - | - | - | - | - | - | - | - |
| CAMP Test | *Staphylococcus aureus* | 6 | 23.07 | 1 | 3.84 | - | - | - | - | - | - |
| | *Rhodococcus equi* | - | - | - | - | - | - | - | - | - | - |

| Tests \ Species | | *L.monocytogenes* | | | | *L. innocua* | | *L. gray* | | *L. welshi-meri* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | serotype 1a | | serotype 4b | | | | | | | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % |
| Mobility | | 6 | 23.07 | 1 | 3.84 | - | - | - | - | - | - |
| Oxidase Test | | - | - | - | - | - | - | - | - | - | - |
| Catalase Test | | 6 | 23.07 | 1 | 3.84 | 16 | 61.53 | 1 | 3.84 | 2 | 7.69 |
| Acid from: Glucose | | 6 | 23.07 | 1 | 3.84 | 16 | 61.53 | 1 | 3.84 | 2 | 7.69 |
| Trehalose | | 2 | 7.69 | 1 | 3.84 | 2 | 7.69 | - | - | - | - |
| D-Mannitol | | - | - | - | - | - | - | - | - | - | - |
| D-Manose | | 6 | 23.07 | 1 | 3.84 | 16 | 61.53 | - | - | 2 | 7.69 |
| L-Rhamnose | | 6 | 23.07 | 1 | 3.84 | 1 | 3.84 | - | - | 1 | 3.84 |
| D-Xilose | | - | - | - | - | 2 | 7.69 | - | - | - | - |
| Serological identification | *L. m.* 1a | 6 | 23.07 | - | - | - | - | - | - | - | - |
| | *L. m.* 4b | - | - | 1 | 3.84 | - | - | - | - | - | - |

*L.m. = L.monocytogenes*

***Table 3***

**Incidence of *Listeria* species from food products (No. / %)**

| Products \ Species | | *L.monocytogenes* | | | | *L.innocua* | | *L.gray* | | *L.welshi-meri* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | serotype 1a | | serotype 4b | | | | | | | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % |
| Raw minced meat (pork and beaf) | | 1 | 3.84 | - | - | 7 | 26.92 | - | - | - | - |
| Paste of Romanian sausages (highly spiced sheep and porc meat balls) | | 1 | 3.84 | - | - | - | - | - | - | - | - |
| Fresh sausages | | 3 | 11.53 | - | - | 3 | 11.53 | - | - | - | - |
| Muscular tissue of | horse | - | - | - | - | 1 | 3.84 | - | - | - | - |
| | pork | - | - | - | - | 2 | 7.69 | - | - | - | - |
| | beef | - | - | - | - | - | - | 1 | 3.84 | - | - |
| Bacon in processing | | - | - | - | - | 1 | 3.84 | - | - | - | - |
| Smocked chop | | 1 | 3.84 | - | - | - | - | - | - | - | - |

| Species / Products | *L.monocytogenes* serotype 1a | | *L.monocytogenes* serotype 4b | | *L.innocua* | | *L.gray* | | *L.welshi-meri* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % | No. | % |
| Pork shoulder blade | - | - | - | - | 1 | 3.84 | - | - | - | - |
| Poultry carcass | - | - | 1 | 3.84 | - | - | - | - | - | - |
| Romanian pressed cheese | - | - | - | - | 1 | 3.84 | - | - | - | - |
| Powder milk | - | - | - | - | - | - | - | - | 2 | 7.69 |
| TOTAL STRAINS | 6 | 23.07 | 1 | 3.84 | 16 | 61.53 | 1 | 3.84 | 2 | 7.69 |

## 3. CONCLUSIONS

1. By implication in the public health, the contamination of food *by L. monocytogenes* raises an important economic problem concerning the food industry. The presence of *Listeria* spp. proved to be a useful indicator during all the stages of the food processing chain.

2. The high incidence of *Listeria* spp. in raw meats can be attributed to fecal contamination during evisceration, or to food handlers.

3. *L. monocytogenes* 1a, the serotype circulating in Romania, was identified in both raw meat and meat products.

4. *L. monocytogenes* 4b, the serotype wide spread in West-Europe, was detected in poultry carcass, imported from Italy.

5. *L. innocua* was the most common species in raw meat, while other *Listeria* specia were less frequently.

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# THE CHARACTERIZATION AND IDENTIFICATION OF SOME MICROORGANISMS ISOLATED FROM THE MEAT OF THE FOOD SNAIL *HELIX POMATIA*

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**Key words**: total germs number, food snail, *Helix pomatia*

**SUMMARY**

Lately, the food snail Helix pomatia is very appreciated in our country. Many people raise it in farms and they also export the snails. Still, their microbiology has not been properly studied in Romania until now. This study's purpose was to isolate microorganisms from the snails meat on different media, to observe the colonies morphological aspects and to identify the isolated species.The colonies aspects were various, according to the medium on which they have grown. The predominant bacteria that we have identified in the snails meat belong to the genus: Citrobacter, Pseudomonas and Enterobacter. On only one medium for pathogenic bacteria, Chromogenic Listeria Agar, we could notice the growth of colonies, identified as Listeria monocytogenes and Listeria innocua. On the Sabouraud medium two species of fungi have grown: Cladosporum and Chrysosporium. Further researches are required in order to establish the influence of these microorganisms on the consumer's health.

Food snails *H. pomatia* meat has always been highly valued for its dietetic and nutritive properties. Lately, in our country, many people began to organize farms in which they raise this specie. The snails' meat is usually exported. Still, in our country, the microbiology of the food snails has not been properly studied, unlike in other countries, where the microbiology of terrestrial gastropods is studies by several authors (for example, Serrano S. et al, 2004). Consequently, the aims of the present paper are: to isolate microorganisms from the snail's meat on several media; to observe the morphological aspects of the formed colonies and to identify the isolated species.

## 1. MATERIAL AND METHODS

The research took place in the following steps:

Step 1 – We collected for our study 15 food snails *Helix pomatia* from a farm.

Step 2 – After removing the shell, the snail foot was decontaminated in alcohol for one minute and then serial decimal dilutions with Brain Heart Infusion broth were prepared, according to the method mentioned

by Bondoc I. and Sindilar E.V. (2002). The dilutions were incubated at 37°C, for 24 hours.

Step 3 – Plates with the foolowing media were then incoulated with 10 µl of the $10^6$ dilution: Agar with sheep bood, MacConkey with Crystal Violet, XLD Agar, Campylobacter Selective Blood Free Agar, Chromogenic Listeria Agar and Sabouraud medium for the isolation fo fungi were then inoculated with 10 µl of the $10^6$ dilution. The media were incubated at 37°C for 24 hours.

Step 4 – We observed the morphological aspects of the formed colonies after 24, 48 and 72 hours.

Step 5 – The species have been identified using the API galleries with automatic reading.

## 2. RESULTS AND DISCUSSIONS

The dimension of the colonies on the Blood Agar varies from punctiform colonies to big, grey colonies with a mucuos aspect.

Some of the colonies present α-hemolyze, other colonies present a small area of hemolyze and others present β- or double hemolyze.

Some colonies are pigmented in citrin yellow, in brown-green, salmon pink or red.

On the MacConkey Agar, most of the colonies are lactose negative after 24 hours. After 48 hours, some of them have a yellow aspect which proves their slow capacity for lactose fermentation.

On the XLD Agar, two types of colonies have formed: yellow, lactose-pozitive colonies and pink, xilose- pozitive colonies.

On the Chromogenic Listeria Agar, on 6 out of 15 samples, we observed the growth of blue-green colonies plus a halo and the growth of blue-green colonies with no halo.

We had no colonies formed on the other media for pathogenic bacteria.

After 5 days, on the Sabouraud-gentamicin-cloramphenicol agar plates we could notice at only one snail, the appearance of two colonies (a black, fluffy one and a white, fluffy one) on the culture made from the shell and of one white, fluffy colony, which had a brown base after 7 days and which was intensively adherent to the medium.

We used the Gram-stain for all the types of colonies. The results are in table 3.

The identification of the Gram-negative bacilli has been made on API galleries with automatic reading.

***Table 1.***

**The colonies aspects and identifications on Bood Agar and on MacConkey Agar**

| Morphology on Blood Agar | Morphology on MacConkey Agar | Morphology on Gram-staining | Identification |
|---|---|---|---|
| - | Big, pink, lactose-positive colonies | Gram-negative bacilli | *E. coli* |
| - | Big colonies, with a central lactose-positive point | Gram-negative bacilli | *Ent. cloacae* |
| - | Big, pink, lactose-positive colonies | Gram-negative bacilli | *Citr. braakii* |
| - | Big, lactose-negative colonies | Gram-negative bacilli | *Morg. morganii* |
| - | Opaque colonies | Gram-negative bacilli | *Ent. amnigenus* |
| - | Big, opaque, lactose-negative colonies | Gram-negative bacilli | *Citr. koseri* |
| Viscous, salmon pink colony | - | Gram-negative, polymorphous bacilli | *Pseudomonas putrefaciens* |
| Colony with a narow haemolytical zone, with a brown-green pigment and irregular edges | Lactose ± | Gram-negative, polymorphous bacilli | *Pseudomonas pseudoalcaligenes* |
| Grey, not haemolytical colonies, with a mucous aspect | Lactose-negative | Gram-negative, bipolar stained bacilli | *Pseudomonas pseudomallei* |
| Grey, mucous colonies, from small to very big ones | Lactose-negative | | *Pseudomonas putida* |

The colonies aspects on Sabouraud and on Listeria Chromogenic Agar are in the next two tables:

***Table 2.***

**Colonies aspects and identifications on Sabouraud medium**

| Morphology | Identification |
|---|---|
| White, fluffy colony, with a brown base | *Cladosporum spp* |
| Black, fluffy colony, appeared after 7 days | *Chrysosporium spp* |

***Table 3.***

**Colonies aspects and identifications on Chromogenic Listeria Agar**

| Morphology | Identification |
|---|---|
| Blue-green colonies plus halo | *Listeria monocytogenes* |
| Blue-green colonies, no halo | *Listeria innocua* |

Predominant in our samples were the bacteria belonging to the genus *Enterobacter, Citrobacter* and *Pseudomonas*. We identified two subspecies belonging to the genus *Enterobacter: Ent. cloacae* and *Ent. amnigenus*, two subspecies belonging to the genus *Citrobacter*: *Citr. braakii* and *Citr. koseri* and four subspecies belonging to the genus *Pseudomonas: Pseud. putrefaciens, Pseud. pseudoalcaligenes, Pseud. pseudomallei* and *Pseud. putida.*

*E. coli* was identified in only two samples of the snail's meat.

The formed colonies on the Chromogenic Listeria Agar were identified as *Listeria monocytogenes* and *Listeria innocua*.

Two species of fungi were identified on the Sabouraud medium: *Cladosporum spp* and *Chrysosporum spp.*

Our researches continue, in order to establish the influence of these species on the consumer's health.

## 3. CONCLUSIONS

3.1. The morphological aspect of the colonies isolated from the snails meat varies, according to the medium on which they have grown.

3.2. Most of the bacteria isolated from the snails meat belongs to the genus: *Citrobacter, Enterobacter* and *Pseudomonas.*

3.3. On Chromogenic Listeria Agar, two types of colonies have grown, identified as *Listeria monocytogenes* and *Listeria innocua.*

3.4. Two species of fungi have grown on Sabouraud: *Cladosporum* and *Chrysosporium*.

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# FACE-TO-FACE AND ONLINE PROFESSIONAL COMMUNITIES FOR VETERINARIANS AND VETERINARY STUDENTS – A FOCUS GROUP STUDY

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**Key words:** Web 2.0, face-to-face communities, on-line communities, focus group, veterinarians and students

**SUMMARY**

Web 2.0 is a relatively new concept and is generally understood as a group of web-based tools that enable collaborative online activities and information sharing. Wikis, discussion boards and podcasts are some of the most popular tools and are increasingly used in medical education. An online professional community, NOVICE, is being developed to support the use of Web 2.0 in veterinary informal lifelong learning. This paper presents a focus group study conducted to investigate veterinary students' and veterinarians' perceptions towards, and experiences of, face-to-face and online professional communities. Ten students and 10 veterinarians participated in the focus groups. They answered questions and entered into discussions that aimed to provide information about current participation in both types of communities, reasons for participation, activities undertaken, barriers to participation, challenges encountered, and support required for participation. Students and veterinarians reported that they are members of several face-to-face and online communities. Veterinarians take part in a larger number of communities than students and both groups participate in more online communities. Both groups had several similarities relating to the activities in face-to-face communities with the social aspect being important and both found online communities useful for information gathering. The main barriers to participation in both types of community were cost and time. Students were sometimes reluctant to contribute in a professional network with veterinarians. Participation in online communities was also sometimes hindered by technical issues, a lack of technical knowledge and concerns about erroneous information. Financial support of the employer or faculty was frequently mentioned in relation to participating in face-to-face communities; technical assistance was required for online communities. The results of the study will be used to inform the development of the NOVICE online professional community.

Web 2.0 is the new face of the internet and although increasingly used, the term is not familiar to all and can be difficult to explain. The term was brought into regular use after O'Reilly's conference [11] and is usually understood as the new forms of web-based tools that promote sociability, creativity and collaboration rather than more traditional webpages where large numbers of people read or 'consume' content created by a few. Web 2.0 tools are designed to support a more

personalised, interactive, real-time internet experience, enabling users with minimal technical knowledge to create, distribute and share content. Some of the more common examples are wikis, blogs, discussion boards (forums), podcasts and social-networking sites [12].

Although Web 2.0 is often associated with Wikipedia and social networks such as Facebook, the benefits of the interactive web-based tools are beginning to be recognized in education including medicine [1, 2] and pharmacy [3]. Web 2.0 tools are being used as an integral part of doctors' information gathering [6] and provide the opportunity, if we choose to take it, to collaboratively create online repositories (medical wiki) which would support clinicians working and learning together [5]. Online environments transcend some of the barriers to face-to-face meetings including distance and time, giving the learner more flexibility 'anytime, anyplace' and allowing open discussion [1]. Paradoxically, the disadvantages are directly linked with this openness. Collaborative web pages can be altered, the reliability of the content cannot be guaranteed and the lack of security of data can be a serious issue e.g. when patients can access a doctor's personal information on Facebook [9]. Therefore, awareness of the issues, careful monitoring and efforts to provide a safe digital environment become an integral part of these communities.

Veterinary schools and practices have been taking advantage of computer aided learning resources for some years e.g. the CLIVE project [4] and successful computer-based educational projects developed in EU higher education have provided useful models for both undergraduate and postgraduate veterinary students [13]. There is an increasing move towards e-CPD to provide an online learning environment and support for continuing professional development [14]. There has been an increasing prevalence of information and communication technology (ICT) in veterinary education [15] as it can complement traditional resources and support undergraduate and continuing professional development. Some of the most recent advances utilise Web 2.0 tools e.g. WikiVet, which provides free access to an increasing volume of online content [17].

The 'Network Of Veterinary ICt in Education' (NOVICE) is an EU funded collaboration between five European veterinary schools (in Bucharest, Utrecht, Hannover, London and Budapest). The project aims to develop a web-based professional network for veterinarians and veterinary students (as well as ICT and veterinary educationalists) providing a range of Web 2.0 tools to support informal lifelong learning [10]. The first part of the project, and subject of the present study, was to

investigate and compare the use of face-to-face and online communities by veterinarians and students.

## 1. MATERIAL AND METHODS

Focus groups were used to determine the experiences and perceptions of veterinary students and veterinarians towards face-to-face and online professional communities. A focus group typically involves 5 – 10 people with an accompanying facilitator who raises pre-determined, but somewhat flexible, questions and topics which are then discussed [7]. A key advantage of the focus group method of data capture is the interaction between the members [16], which allows for in depth discussion and further exploration of the key issues that emerge. Therefore, focus groups can often give more insight than interviews [8]. They can also be used to guide survey development and refine research questions.

Ten students and 10 veterinarians volunteered to participate in the focus groups held at the Faculty of Veterinary Medicine, Bucharest and were recruited through personal contact by one of the authors (EC), who was also the focus group facilitator. Ethics approval for the study was granted by the faculty's ethics committee.

A set of predetermined questions were used to cover the key topics and aimed to provide information about:

1. Which face-to-face communities our participants have joined
2. Which online communities our participants have joined
3. Reasons for participation
4. Activities when participating in both types of community
5. Barriers to participation
6. Challenges encountered
7. Support required

At the beginning of the focus group a short PowerPoint presentation (10 minutes) was delivered by the facilitator, which included an explanation of Web 2.0 and provided examples of some of the most popular tools (including discussion boards, wikis and blogs). This ensured that even those who had not heard of the term before were able to take part in the discussions about face-to-face and online communities. The focus group facilitator took notes and the sessions were audio recorded and transcribed. The transcripts (qualitative data) were analysed to identify the major themes under each topic heading. Comparisons were made between both categories of communities in terms of motivation and barriers, as well as identifying differences and

similarities between the students' and veterinarians' activities and opinions.

## 2. RESULTS AND DISCUSSIONS

Two focus groups were held with students, in total seven females and three males participated. The students ranged from Year 2 to Year 6. Due to difficulties arranging a time when all ten veterinarians could meet, the facilitator conducted four smaller focus groups with two or three participants in each. There were five females and five males in total. They were clinicians (small animal, farm animal, equine or exotic) and a PhD student.

The students and veterinarians were all members of one or more face-to-face and online communities. As can be seen in Table 1, veterinarians take part in a larger number of communities, both face-to-face and online, than students. Both groups were members of more online than face-to-face communities.

***Table 1***

**Participants' membership of face-to-face versus online communities**

| | | Face-to-Face Communities | Online Communities |
|---|---|---|---|
| Veterinary Students | Number Mentioned | 3 | 11 |
| | Examples | • Association of Students in Veterinary Medicine<br>• Paco Foudation | • IVIS<br>• fmvstudents.blogspot.com<br>• gigapedia.com |
| Veterinarians | Number Mentioned | 12 | 25 |
| | Examples | • Association of Small Animal Veterinarians<br>• General Association of Romanian Veterinarians | • INFOVETERINAR<br>• WIKIVET<br>• THEHORSE.COM |

During the focus groups, motivation for participating in these communities as well as the activities undertaken and the benefits of membership were discussed. Table 2 displays these positive associations with face-to-face and online communities for students and veterinarians.

***Table 2***

**Activities within communities and their benefits to the participants**

| | Face-to-Face Communities | Online Communities |
|---|---|---|
| Veterinary Students | • Participate in events and meetings and improve personal professional development while keeping up to date with new developments<br>• Improve my CV<br>• Collaborative activities (fund raising, organizing events, writing articles)<br>• Improve the relationship between students and teachers<br>• Socialise, making and maintaining contacts, plus meet people from different parts of the country/ abroad | • Gathering information about veterinary issues and for pleasure (articles, images, audio)<br>• Write content on student association site<br>• Make and maintain contacts<br>• Discussions with experienced vets |
| Veterinarians | • Requirement for veterinarians (Romanian College of Veterinary Surgeons)<br>• Participate in periodical lectures, keynotes, conferences and annual symposium or congress<br>• Receive community's journals and access new information<br>• Practical courses – learn<br>• Gain information about jobs, workshops<br>• Translate community's articles and announcements into Romanian<br>• Exchange ideas and experience<br>• Socialise | • Gathering information about veterinary issues (films, articles, images)<br>• Write and correct content (Wikipedia)<br>• Make and maintain contact with friends and specialists<br>• Read and reply to blogs<br>• Exchange experience, upload and download professional information<br>• Give feedback on cases |

The students and veterinarians highlighted the benefits of taking part in both types of professional communities as gaining and sharing information and making and maintaining contacts, albeit in slightly different ways. The social element of face-to-face communities was recognized by veterinarians and students alike:

"The meetings with colleagues are always pleasant and useful considering networking opportunities" [veterinarian].

The students noted that their relationship with other students, teachers and veterinarians improved within a community and

professional, scientific and organizational abilities were considerably improved:

"I used to think that participating in these activities was a waste of time and energy, but spending more time in this association has proved to me that our lives will improve as result of the communication and the information available" [student; face-to-face].

Students and veterinarians saw the online communities as a viable alternative to face-to-face meetings overcoming problems relating to distance, lack of support from colleagues and employers, time and cost:

"The obvious reason: I get information easily. Maybe for some of us it is easier to open a laptop than open a book" [student].

"Idealistically speaking, a big online community would be convenient for all of us. Being there, you have the chance to ask and get answers from many people." [veterinarian]

There are however difficulties that may be faced within communities – some can be overcome, and are known/classified as challenges, while some prevent any participation and are known/classified as barriers. Many participants could name professional communities that they were aware of but did not participate in, due to these barriers, for example the Association of Small Animal Veterinary Practitioners (Asociatia Medicilor Veterinari pentru Animale de Companie - AMVAC). Table 3 highlights both the challenges and barriers faced when trying to participate in a professional community.

***Table 3***

**Challenges and barriers to participation in professional communities**

| | Face-to-Face Communities | Online Communities |
|---|---|---|
| Veterinary Students | • Lack of spare time - full curriculum, job<br>• Financial: costs of membership, fund raising<br>• Difficulties within the community – lack of interest, internal disputes, lack of volunteers for tasks, poor communication, students not accepted<br>• Lack of trust in some communities<br>• The level of knowledge too high for students in the first academic years<br>• Narrow area of interest (specialists)<br>• Lack of publicity - limited newsletter and invitations to the members only<br>• Bureaucracy<br>• Finding new members | • Uncomfortable in a professional community for vets - lack of self confidence to contribute<br>• Insufficient knowledge or exclusive terminology<br>• The level of knowledge too specialized for students<br>• Information too general<br>• Uncertain or erroneous information<br>• Low quality of some materials<br>• Cost related barriers<br>• Lack of purpose in education for some online communities (Unyka, Twitter)<br>• Too complex design of the site |
| Veterinarians | • Lack of time and timing- work schedule<br>• Costs of membership, accommodation, travel<br>• Bureaucracy (complicated registration)<br>• Limited acceptance of new members (e.g. from other countries)<br>• The high level of discussions and expertise Lack of employer support<br>• Lack of publicity of some events, organization problems of meetings<br>• Lack of transparency of some face-to-face professional communities<br>• Limited places in workshops | • Lack of spare time and information<br>• Sites not 'user friendly'<br>• Cost related problems (downloading text, picture, films)<br>• Restriction of important files, pictures (cost limited downloads)<br>• Low quality of the materials (DVD with bad image and low sound)<br>• Lack of publicity of some events<br>• Bureaucracy (complicated registration) |

The challenges and barriers most frequently mentioned by both groups and towards both face-to-face and online communities were time and cost:

"The hardest situation is when I have to make time between job and school obligations, so the activities of the association are sometimes neglected" [student]

"There are so many online communities, but my spare time resource ran out a long time ago. That's why I don't like the idea of joining other online communities." [veterinarian]

"Registration and membership dues, accommodation and transportation. Sometimes this cost does not justify the importance of the event" [veterinarian].

Other significant challenges for the students were feeling worthy of contributing, partly due to a lack of understanding the professional terminology used. They were also concerned about erroneous information:

"We cannot always be sure whether the posted information is based on research and detailed observation. A research paper is reviewed prior to publication and provides secure information" [student].

For veterinarians, challenges to online communities involved technical issues related to the website's design:

"Usually, I'm a patient person, but the layout of some pages gives me a headache. I can hardly find what I'm interested in" [veterinarian]

Like the students, they also have issues relating to trusting the people who post information. Further to this, direct traditional contact though phones and e-mails are preferred to blogs or online communities when the people involved are known to each other.

Participation in any professional community, requires support, for example financial, technical assistance and facilities for being informed (Table 4). For the students' group, financial support seems to be an important issue. They also have some reservations about providing personal information and the purpose of online communities:

"I would like that some information I've provided previously to be stored securely. I strongly believe that the membership of online community must be sustained by the membership of a face-to-face community." [student]

Veterinarians concur regarding the need for financial support for successful participation in professional communities:

"The costs for online access on database are sometimes prohibitive for the Romanian vets" [veterinarian])

Further to this, they require technical information for easy access to online communities, and a better promotion of events, workshops and vacancies.

***Table 4***

**Support required for participation in face-to-face and online communities**

| | Face-to-Face Communities | Online Communities |
|---|---|---|
| Veterinary Students | • Transparency of actions, ability to work as a team<br>• Faster access to relevant information<br>• A better publicity of professional communities<br>• Chance to have a good advisor, mentor or tutor (veterinarian)<br>• Easy access to information<br>• Lower taxes or free of charge participation for students<br>Lectures and workshops organized exclusively for students | • Accessible language for students<br>• Technical information - how to use the site<br>• Financial support<br>• Better communication between students and vets<br>• List and links with other online communities<br>• Assure privacy for personal data and information<br>• Support from teachers to join in<br>• Assurance of accurate information |
| Veterinary Veterinarians | • Better support from professional communities<br>• Financial support from employer<br>• Newsletter about vacancies, workshops Downloads free of charge for the members | • More publicity<br>• Broader access to information without prohibitive costs<br>• Easy access (e.g. one password) and technical information<br>• Financial support<br>• Lists and links to important and available online communities<br>• Online lectures<br>• Broad community - diverse specialties |

The focus group meetings resulted in enthusiastic discussions and provided useful information about current participation and activities in both face-to-face and online communities. The findings relating to online communities were similar to those of others: with regard to motivation, Giustini (2006) also reported the importance of discussing openly across geographical expanses; and with regard to barriers, concerns relating to privacy and authenticity of content were particularly highlighted by Boulos *et al.* (2006) and MacDonald *et al.* (2010).

The results of this study will be used in conjunction with findings from focus groups and surveys being undertaken by all five members of the NOVICE project team (based at veterinary schools in Bucharest,

Utrecht, London, Hannover and Budapest). The reasons for participation in online communities will help to direct the design of the NOVICE professional network in order to serve the needs of veterinarians and veterinary students and support their informal lifelong learning. In particular, the technical challenges and concerns about the skills required to use Web 2.0 tools will be factored into the development of the site. A major requirement will be to provide support for new users and those unfamiliar with online networks and the associated interactivity and functionality afforded by Web 2.0. Although participation in any community inevitably requires an investment in time, NOVICE is free to join. Ultimately, the project team aim to design and support a network that will link veterinarians and students across the EU and beyond, and allow the growth of special interest groups and encourage topical discussions and exchange of information.

## 3. CONCLUSIONS

3.1. Veterinarians take part in a larger number of communities than students and both groups participate in more online communities.

3.2. Both groups had several similarities relating to the activities in face-to-face communities with the social aspect being important and both found online communities useful for information gathering.

3.3. The main barriers to participation in both types of community were cost and time. Students were sometimes reluctant to contribute in a professional network with veterinarians.

3.4. Participation in online communities was also sometimes hindered by technical issues, a lack of technical knowledge and concerns about erroneous information.

3.5. Financial support of the employer or faculty was frequently mentioned in relation to participating in face-to-face communities; technical assistance was required for online communities.

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# THE USE OF ULTRASOUND EXAM IN VISUALIZING THE DISTAL INTERPHALANGEAL JOINT ANATOMY IN HORSE

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**Key words:** equine foot, ultrasonography, anatomy, distal interphalangeal joint

## SUMMARY

The clinical importance of distal interphalangeal joint (DIPJ) resides in its role in foot biomechanics and lameness. The equine foot is an anatomical part of the body that was considered hard to be imaged by ultrasound. Ultrasonography was found particularly useful in horses presenting foot lameness without significant radiographic findings (Denoix and Audigie, 2004). Our objective was to develop a systematic echographic approach of DIPJ and to present the accuracy of the ultrasound exam in the diagnosis of ligament and tendon injuries in the foot.
In order to obtain a complete examination of the distal interphalangeal joint, five different approaches have been used: dorsal, collateral lateral and medial, palmar and distal or transcuneal. For each approach, both longitudinal and transverse sections were realized. A standoff pad was used for the dorsal and abaxial approaches. The ultrasound of the foot has been done with a non-portable machine *Aloka Prosound Alpha 10* (Aloka Co. Ltd., 6-22-1 Mure, Mitaka-shi, Tokyo, 181, Japan). Linear, convex and microconvex multifrequence transducers, with a frequency varying between 5.0 to 10.0 MHz have been used. This technique allowed us to image almost all the components of DIPJ, with the specification that it presents some limitations in examining the articular surfaces and the deep part of the bone in the case of the distal phalanx and the distal sesamoid bone.

The distal interphalangeal joint (DIPJ), also known as coffin joint, represents an articular complex between the middle phalanx the distal phalanx and the sesamoid bone. Imaging techniques of this region, available to the equine practitioners include radiography, ultrasonography, thermography, nuclear scintigraphy, computed tomography and magnetic resonance imaging (Redding, 2009). Ultrasonography was found particularly useful in horses presenting foot lameness without significant radiographic findings (Denoix and Audigie, 2004). The major advantage of this procedure is its real-time, dynamic assessment (Denoix, 1996a). In order to obtain good quality images and to understand the ultrasonographic anatomy, precise knowledge of descriptive, topographical and microscopic anatomy is necessary. Reference ultrasound images are undoubtedly necessary for a correct interpretation of the clinical ultrasonograms in order to asses an accurate diagnosis and to avoid misinterpretation.

Our objective was to develop a systematic echographic approach of DIPJ and to present the accuracy of the ultrasound exam in the diagnosis of ligament and tendon injuries in the foot.

## 1. MATERIALS AND METHODS

Ten healthy horses, with no history of lameness and without any clinical sign like local swelling or joint distension have been ultrasounded in order to obtain reference images of the equine foot. The horses belong to CIRALE, France, and they were examined here.

The examined region was clipped with a #40 fine blade electric clipper. The clipped surface was washed with warm water and a sponge. A standoff pad was used for the dorsal and abaxial approaches. The coupling acoustic gel (Sonogel, Germany) was used. The ultrasound of the foot has been done with a non-portable machine *Aloka Prosound Alpha 10* (Aloka Co. Ltd., 6-22-1 Mure, Mitaka-shi, Tokyo, 181, Japan). Linear, convex and microconvex multifrequence transducers, with a frequency varying between 5.0 to 10.0 MHz have been used. The most often used frequency was 7.5 MHz. In order to obtain a complete examination of the distal interphalangeal joint, five different approaches have been used: dorsal, collateral lateral and medial, palmar and distal or transcuneal. For each approach both longitudinal and transverse sections were realized.

## 2. RESULTS AND DISCUSSIONS

*Articular Surfaces*

The second phalanx (Fig.1, no.10) is represented by a slight concave hyperechogenic line, which corresponds to the condyles and the intercondylar groove of the distal extremity of this bone. The articular cartilage of the middle phalanx (Fig.1, no.11) has a well defined anechogenic aspect and lies between the hyperechogenic subchondral bone and echogenic articular space. The only part from the distal phalanx that can be imagined with this approach is *processus extensorius* (Fig.1, no.12a) which appears as a hyperechogenic line. The distal sesamoidian bone (Fig.3, no.8) appears as a short hyperechogenic line situated on the dorsal aspect of the deep digital flexor tendon and on the plantar one of the distal phalanx. The dorsal border of the flexor surface of the distal sesamoid is visible within the most distal approach between the heel bulbs and is represented by a hyperechoic line with an acoustic shadow, orientated dorsally.

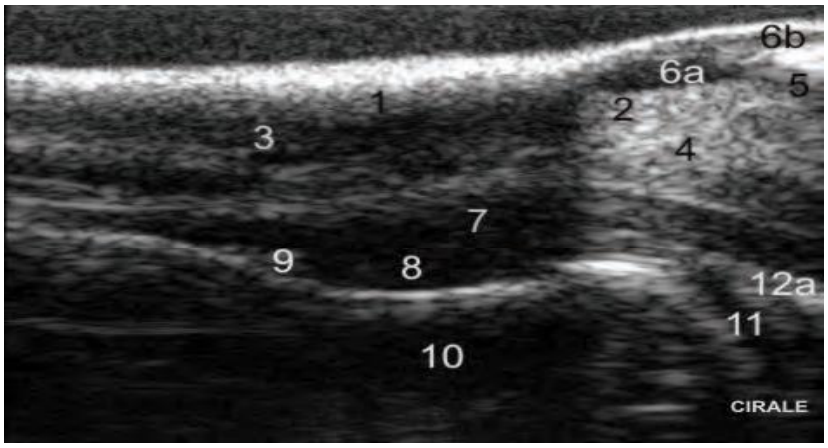


**Fig. 1. Sagittal ultrasonographic section of the dorsal aspect of the equine foot**
**1. Skin. 2. Corium limbi. 3. Subcutaneous tissue. 4. Pulvinus coronae. 5. Corium coronae. 6a. Periople. 6b. Stratum externum. 7. Dorsal digital extensor tendon. 8. Dorsal recess of the distal interphalangeal joint. 9. Articular capsule. 10. Middle phalanx (P2). 11. Articular cartilage of P2. 12. Distal phalanx (P3). 12a. Extensor process.**

*Joint Capsule*

The joint capsule is very tight dorsal and lateral and blended with the dorsal digital extensor and ligaments respectively. The joint synovial membrane presents five different recesses: dorsal, two collaterals, proximal palmar recess and distal palmar recess (Damian, 2001). Only the dorsal and palmar recesses can be imaged ultrasonographically.

*The dorsal recess of the distal interphalangeal joint* (Fig.1, no.8) is anechogenic and is situated between the dorsal digital extensor tendon and the articular cartilage of the middle phalanx. The dorsal recess is delimited by the distal interphalangeal articular capsule (Fig.1, no.9) which is attached around the margins of the articular surfaces. It has an irregular shape and a moderate echogenic aspect. Dorsally it is blended with the dorsal digital extensor tendon's fibers. The synovial membrane is not visible under normal conditions.

*The palmar recess of coffin joint* is greatly expandable, 25 to 30 ml in volume (Bowker, 2003) and is divided into a large proximal palmar recess and a smaller distal palmar recess. The proximal palmar recess is anechoic and is situated between the palmar surface of the middle phalanx and the podotrochlear bursa (Fig.3, no.7). A fibroelastic sheath separates it from the distal recess of the digital sheath. This recess is also separated from the podotrochlear pouch by an echoic structure, the collateral (proximal) sesamoidean ligament (Fig.3, no.6). The proximal

palmar pouch almost surrounds the collateral sesamoidian ligaments and the lateral limits are expanding till the abaxial surfaces of the ungual cartilages. The distal palmar recess is extending between the distal sesamoid bone and the distal phalanx. The palmar limit is represented by the dorsal aspect of the distal impair sesamoidian ligament, which also separates the palmar distal recessus from the podotrochlear bursa.

*Collateral Ligaments of the Distal Interphalangeal Joint*

There is a medial and a lateral collateral ligament. They are short and have a triangular aspect. The proximal insertion is in the depressions on either side of the lower part of the second phalanx, and the distal insertion is in the depressions on either side of the extensor process of the distal phalanx. Both collateral ligaments contain thick and parallel fibers bundles that are orientated approximately vertically, but obliquely compared to the phalangeal axis (Denoix, 1998b). Due to their obliquity the scanhead is difficult to be moved strictly parallel to the fibres bundles and their appearance is heterogeneously echogenic. The optimum echogenicity is obtained when the ultrasound beam is perpendicular to the examined tissue (Denoix, 1996a; Denoix et al., 1996b; Busoni and Denoix, 2001).

For the visualization of the distal part and distal insertion fossa of the collateral ligament, a 7.5 or 10.0 MHz sector array can be used (Denoix, 2002). The proximal part of the collateral ligament is situated in the concave collateral fossa of the middle phalanx, which is represented by a hyperechogenic line (Fig.2, no.6). The distal part of this ligament is difficult to be scanned because it is located deep to the hoof wall. On transverse section the collateral ligaments of the distal interphalangeal joint (Fig.2, no.4) have an oval aspect and a homogenous echogenic structure. On the palmar aspect of this transverse section an anechogenic structure is imaged near the palmar border of the collateral ligament. This is the ungular cartilage (Fig.2, no.5). Both medial and lateral collateral ligaments should be examined, so that any difference of size and echogenicity that could indicate a lesion will be noted. Size and echogenicity of both collateral ligaments are also evaluated comparatively in the symmetrical limbs.

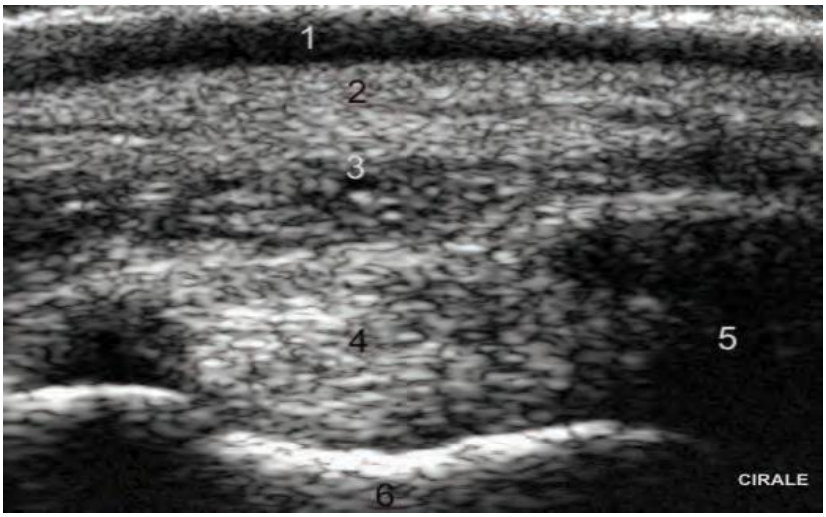


**Fig. 2. Transverse ultrasonographic section of the collateral aspect of the foot**
**1. Periople. 2. Corium limbi. 3. Pulvinus coronae. 4. Collateral ligament. 5. Ungular cartilage. 6. Middle phalanx.**

*Sesamoidian Ligaments*

*a) The Collateral Sesamoidian Ligament - Suspensory Sesamoidean Ligament*

These ligaments are fibro-elastic structures. They consist of a thick, horizontal band that is disposed along the palmary edge of the distal sesamoid bone, also known as sagittal union or proximal collateral sesamoidean ligament (Denoix, 2009) and two vertical arms that extend from the extremities of this band on each side of the middle phalanx, till the distal end of the proximal phalanx, just dorsal to the collateral ligament of the pastern joint (Floyd, 2007). The horizontal band prolongs the facies flexoria of the navicular bone and forms the distal scutum. Between the distal scutum and the deep digital flexor tendon, the podotrochlear bursa is interposed. The collateral ligament of the distal sesamoid bone is interposed between the podotrochlear bursa palmary and the proximal palmar recess of the distal interphalangeal joint, dorsally. This ligament corresponds to the distal scutum and appears like a short echoic band that is continued medial and lateral with the collateral ligaments of the distal sesamoid bone (Fig.3, no.6). The collateral ligaments of the distal sesamoid bone are less echogenic than the horizontal part of ligament (proximal ligament) and their fibers have an oblique orientation. It is very important to compare the size and echogenicity of the medial and the lateral collateral sesamoidean

ligaments from the same limb, but also with the ones from the symmetrical limb, in order to identify the lesions.

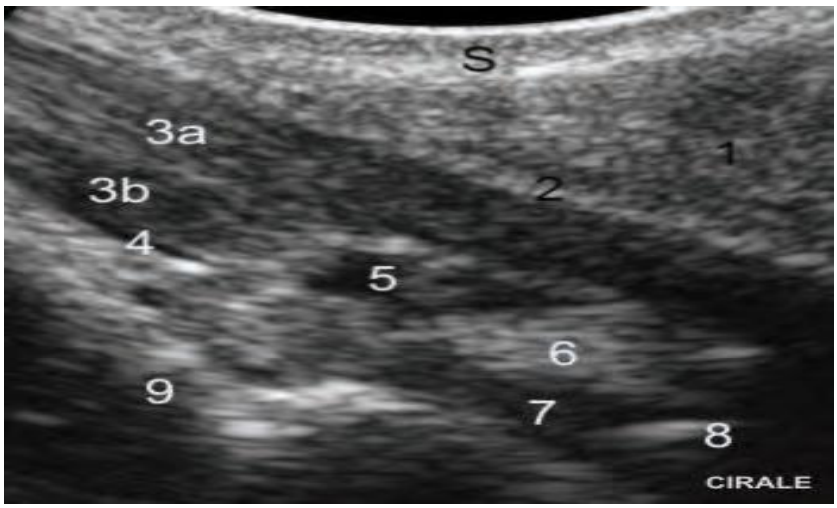


**Fig. 3. Longitudinal ultrasonographic section of the foot using palmar approach**
**A. Sagittal section; B. Parasagittal section**
**1. Digital cushion. 2. Distal digital annular ligament. 3a. Fibrous part of the deep digital flexor tendon. 3b. Fibrocartilaginous part of the deep digital flexor tendon. 4. Digital sheath. 5. Podotrochlear bursa, proximal recess. 6. Collateral sesamoidean ligament. 7. Proximal palmar recess of the distal interphalangeal joint. 8. Distal sesamoidean bone. 9. Middle phalanx. S. Skin.**

*b) The Distal Impair Sesamoidian Ligament*

It is a short, broad, strong ligament that attaches the distal margin of the distal sesamoid bone to the distal phalanx, across the entire width of the joint surface at this level. This ligament separates the coffin joint capsule (the distal palmar recess) from the navicular bursa in this region. In sagittal and parasagittal sections the distal impair sesamoidean ligament (Fig.4, no.5) has a triangular aspect, is echogenic and occupies the space between the distal phalanx, the distal aspect of the distal sesamoid bone and the dorsal aspect of the deep digital flexor tendon. Both proximal and distal insertions of this ligament can be examined ultrasonographically.

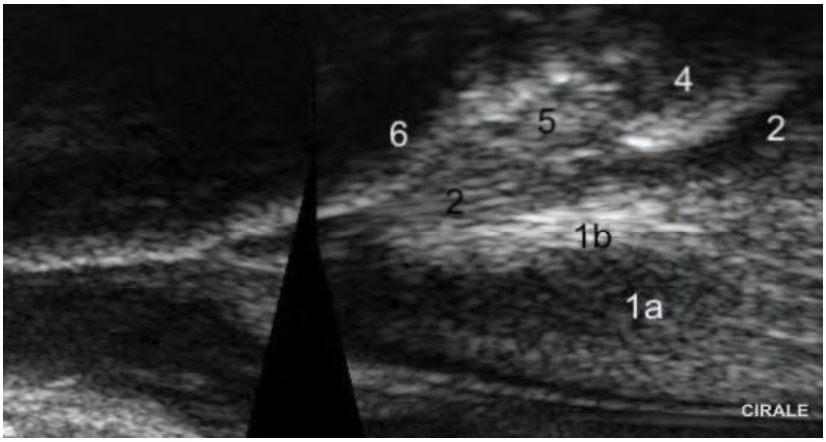


**Fig. 4. Longitudinal ultrasonographic section of the foot using transcuneal approach 1a.Fibroelastic part of the digital cushion. 1b. Fibrous part of the digital cushion. 2. Distal part of the deep digital flexor tendon. 4. Distal sesamoid bone. 5. Distal impair sesamoidean ligament. 6. Palmar aspect of the distal phalanx.**

## 3. CONCLUSIONS

3.1. The ultrasound exam allowed us to examine all the major components of the distal interphalangeal joint in the equine foot.

3.2. Five different approaches were necessary for a complete ultrasound examination of the distal interphalangeal joint, in order to examine the dorsal, abaxial, palmar and distal aspect of this region. For each approach longitudinal and transverse ultrasound section must be obtained.

3.3. In order to identify the modifications of size, shape, echogenicity or architecture, all the examined structures must be compared with the symmetrical one of the same limb, or with the equivalent one of the opposite limb.

3.4. The position and orientation of the probe is crucial for the resolution and quality of the image, but also for the echogenicity. Concordant to specialized literature, the optimum echogenicity is obtained when the ultrasound beam is perpendicular to the examined tissue.

3.5. The articular space, the deep osseous part of distal phalanx and of distal sesamoid bone, as well as the collateral parts of the distal impair sesamoidean ligament can not be well visualized.

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# THE EFFECT OF POLYPHENOLIC EXTRACTS UPON SOME HAEMATOLOGICAL AND BIOCHEMICAL BLOOD PARAMETERS IN RATS WITH ASCITOGENOUS HEPATIC TUMORS

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**Key words**: plant polyphenols, Viscum album, Aristolochia clematitis, Lycopodium clavatum

**SUMMARY**

The purpose of these *in vivo* studies was to follow the effect of some poyphenolic extracts obtained from mistletoe (*Viscum album*), birthwort (*Aristolochia clematitis*) and stag's-horn clubmoss (*Lycopodium clavatum*) upon some hematological and biochemical blood parameters in rats with ascitogenic hepatic tumors.
The evaluation of hematological parameters RBC, HTC, Hb and WBC indicated an improvement of hematopoietic function in rats with ascitogenic liver tumors, the most significant results being obtained in case of stag's-horn clubmoss extract.
The investigation of some biochemical parameters (total proteins, albumins/globulins ratio, ALT, AST and GGT) demonstrated that polyphenolic extracts obtained from mistletoe, birthwort and stag's-horn clubmoss inhibited cytolysis of hepatocytes and improved the values of total proteins and albumins/globulins ratio.

Phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents and range from simple phenolic molecule to highly polymerized compounds (Bravo, 1998). Phenolic compounds exhibit a wide range of physiological properties, such as antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardio-protective, vasodilatory and anticancer effects (Benavente-Garcia *et al.,* 1997; Manach *et al.*, 2005; Middleton *et al.*, 2000; Puupponen-Pimia *et al.*, 2001; Ryoyama *et al.*, 2004; Samman, 1998).

The most studied of these compounds are the green tea polyphenols, particularly catechins, and there are many reports regarding the antitumoral activities of epigallocatechin-3-gallate (Blanko *et al.*, 2003; Gupta *et al.*, 2003).

Studies on apple polyphenols demonstrated that oral administration of a 0.5% crude fraction of an apple polyphenol significantly inhibited the spontaneous lung metastasis of r/m HM-SFME-1 tumor cells, but did not significantly inhibit tumor growth at the site of transplantation. This fraction dose-dependently inhibited the *in vitro* invasion and migration

of the tumor, and inhibited slightly the MMP-9 production and IFN-g plus LPS-augmented VEGF gene expression of the tumor, although non-augmented VEGF gene expression was stimulated in a dose-dependent fashion by the fraction (Ryoyama *et al.*, 2004).

Polyphenols action in cancer prevention and cancer therapy is suggested to be due to antioxidant properties, inhibitory effect on proteasome activity in tumor cell and to protecting effect on DNA from methylation (Chen and Ping Dou, 2008). In fact, they can modulate oxidative tress in cancer cells, thereby affecting signal transduction, activation of redox-sensitive transcription factors and expression of specific genes that influence cell proliferation and apoptosis (Giovannini *et al.*, 2007).

The aim of this study was to observe the effect of polyphenols extracted from mistletoe (*Viscum album*), birthwort (*Aristolochia clematitis*) and stag's-horn clubmoss (*Lycopodium clavatum*) upon some haematological and biochemical blood parameters in rats with ascitogenic hepatic tumors.

## 1. MATERIALS AND METHODS

***Obtaining vegetal extracts***

Dried plant material was extracted with ethanol for 3 hours, allowed to cool and filtered using Whatman no. 1 filter paper. The obtained filtrates were centrifuged at 5000 rpm for 20 min at 5ºC. The filtrates were kept at 4ºC until testing.

***Determination of total phenolics compounds***

The total phenolics content was estimated using Folin-Ciocalteu reagent based assay. To the mixture containing 500 µl plant extract and 4.5 ml of water, 0.2 ml Folin-Ciocalteu reagent was added. The mixture was kept for 5 min at room temperature and then 0.5 ml of 20% $Na_2CO_3$ were added. The mixture was allowed to stand at room temperature for 30 min and then the absorbance at 765 nm was recorded using an UV-VIS-NIR spectrophotometer (Jasco 670). Gallic acid was used as standard for calibration curve.

***Experience animals***

Female rats were inoculated with Walker ascitogenic tumor. This type of tumor induces death of all animals within 14-21 days after intraperitoneal inoculation treatment. Rats were grouped, kept in polyacrylic cages and maintained under laboratory conditions and they were allowed free access to dry pellet diet and water *ad libitum*. All

procedures were carried out in strict accordance with the guidelines prescribed by European legislation for experimental animals.

***Animal assay***

The rats were divided in 5 groups (n = 5). Group A was represented by rats with ascitogenic hepatic tumors, groups B, C and D were represented by rats with ascitogenic hepatic tumors which were administered polyphenols extracted from mistletoe, birthwort and stag's-horn clubmoss, while group E contained healthy rats. Polyphenolic extracts were administered orally (by gavage) using distillated water as vehicle. The rats were monitored for 10 days; on the 10th day, blood was collected under mild anesthesia in order to determine hematological and biochemical parameters.

***Hematological parameters***

The blood samples were collected and investigated for haemoglobin concentration (Hb) by cyanomethaemoglobin method, hematocrit (HTC), red blood cells (RBC) and white blood cells (WBC) counts by haemocytometer method (Choudhri *et al.*, 1997).

***Biochemical parameters***

Blood was collected and the serum was separated at 2000 rpm for 30 minutes. The effects of extracts on total protein, albumins/globulins ratio (A/G), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) were determined.

## 2. RESULTS AND DISCUSSIONS

The obtained results of hematological parameters indicate an improvement of RBC, HTC and Hb values in case of group D treated with stag's-horn clubmoss extract; in case of groups B and C treated with mistletoe and birthwort, the values obtained for RBC, HTC and Hb were lower (table 1).

Total protein parameter was found to be decreased significantly in the cancer control group (group A) when compared with the normal group (group E). Administration of plant extracts led to increased values of total protein as compared with cancer control (table 2).

Thus, the administration of mistletoe, birthwort and stag's-horn clubmoss polyphenols in rats with ascitogenic hepatic tumors produced the increase of albumins biosynthesis and the improvement of A/G ratio. Similar to the other parameters, the best results were obtained in case of group D, treated with polyphenols extracted from stag's-horn clubmoss (table 2).

*Table 1*

**The effect of polyphenols extracted from mistletoe, birthwort and stag's-horn clubmoss on hematological parameters in rats with hepatic tumors (mean values)**

| Group | Plant polyphenolic extract | Polyphenols dose (mg/kg/day) | RBC x $10^6$ / $mm^3$ | HTC (%) | Hb (g/dl) | WBC x $10^3$ / $mm^3$ |
|---|---|---|---|---|---|---|
| A | - | - | 5,1 | 20,8 | 6,7 | 8,2 |
| B | Mistletoe | 50 | 6,1 | 27,2 | 9,1 | 8,9 |
| C | Birthwort | 50 | 5,9 | 27,4 | 9,2 | 8,8 |
| D | Stag's-horn clubmoss | 50 | 7,1 | 29,3 | 9,9 | 8,2 |
| E | - | - | 10,5 | 40,5 | 14,1 | 10,5 |

*Table 2*

**The effect of polyphenols extracted from mistletoe, birthwort and stag's-horn clubmoss on protein biochemical parameters in rats with hepatic tumors (mean values)**

| Group | Plant polyphenolic extract | Polyphenols dose (mg/kg/day) | Total protein (g/dl) | Albumins (%) | Globulins (%) | A/G |
|---|---|---|---|---|---|---|
| A | - | - | 2,7 | 35,8 | 64,2 | 0,56 |
| B | Mistletoe | 50 | 3,2 | 48,3 | 51,7 | 0,93 |
| C | Birthwort | 50 | 3,1 | 46,1 | 53,9 | 0,86 |
| D | Stag's-horn clubmoss | 50 | 4,8 | 55,7 | 44,3 | 1,26 |
| E | - | - | 6,4 | 62,5 | 37,5 | 1,66 |

The obtained results after dosing several enzymes that are considered markers of hepatic cytolysis confirmed the results of protein parameters determinations. The determination of ALT, AST and GGT enzymes activity revealed the decrease in cytolysis process of hepatocytes in rats treated with plant polyphenolic extracts. The lowest enzymatic activities for ALT, AST and GGT were recorded for group D, treated with stag's-horn clubmoss extract (table 3).

*Table 3*

**The effect of polyphenols extracted from mistletoe, birthwort and stag's-horn clubmoss on the activity of some plasmatic enzymes - markers of hepatic cytolysis in rats with hepatic tumors (mean values)**

| Group | Plant polyphenolic extract | Polyphenols dose (mg/kg/day) | ALT (U/l) | AST (U/l) | GGT (U/l) |
|---|---|---|---|---|---|
| A | - | - | 80,1 | 131,6 | 8,7 |
| B | Mistletoe | 50 | 66,5 | 123,6 | 6,6 |
| C | Birthwort | 50 | 65,5 | 119,7 | 6,7 |
| D | Stag's-horn clubmoss | 50 | 51,7 | 92,5 | 5,9 |
| E | - | - | 32,1 | 34,5 | 3,5 |

## 3. CONCLUSIONS

3.1. Rats with inoculated ascitogenic hepatic tumors that were treated with plant polyphenols showed an improvement of hematological parameters compared to untreated control group.

3.2. The investigation of enzymes markers of hepatic cytolysis revealed the decrease of hepatocytolysis process in case of animals treated with plant polyphenols.

3.3. The administration of polyphenolic extracts in rats determined the increase of total proteins and albumins values and also the improvement of albumins/globulins ratio.

3.4. The best results were obtained in case of rats treated with stag's-horn clubmoss extract (group D).

## ACKNOWLEDGEMENTS

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# RESEARCHES REGARDING THE BODY SIZES DYNAMICS DEPENDING ON AGE IN RAINBOW AND SPRING TROUT

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**Key-words:** rainbow trout, spring trout, body length, body weight.

**SUMMARY**

Due to its fast growing rhythm and also the efficient food using coefficient, the rainbow trout, or the American trout as it is called, has become the favorite fish in the consumption salmon fish breeding field. The present paper had in view the study of the body sizes dynamics in rainbow and spring trout, depending on their age. The researches were carried out in Pucioasa trout breeding unit, on the young and adult livestock. The young individuals reached a mean body weight of 38, 29±2, 89 grams and a mean body length of 13, 55± 0,905 cm at one year age and the adult individuals reached the mean body weight of 128, 37 grams and the mean body length of 26,7cm.

## 1. MATERIAL AND METHOD

The research in the present paper was achieved using the data recorded in Pucioasa fish breeding unit, Dambovita County. This fishing unit is located near Ialomita River and it spread over 2, 4 ha and 9240 m of pools. Its capacity consists of 30 tones of consumption trout per year and also 800000 small fish for mountain waters population in the area. The water quality corresponds to the stipulated standards, so they could develop trout's reproduction and rearing. There were carried out measurements and weighing using adequate equipment (ruler, weighing machine, fish meter). There were recorded the noticed values, they there were statistically processed and after that, compared with the ones in the special literature. There were calculated the following variability parameters: the average, its error, the standard deviation and the variability coefficient. The rainbow trout, called also the American trout has the scientific name of Salmo Irideus Gibbons. It was introduced in our country at the beginning of the XX century. It reproduces early, in spring, in March or April, rarely in the first decade of May. Conformingly the development conditions, it reached the sexual maturity at 2-4 years old. Due to its fast growing rhythm it has become the most preferred fish for the salmon breeders.

## 2. RESULTS AND DISCUSSIONS

The table 1 presents the normal growing limits in rainbow trout bred in intensive fishing units. The values are framed per quality categories and they are spread from 1 month old, to three years old and present the body length in centimeters and the body weight in grams, also for reproducers. Following the own researches, we were able to compared the mean values in the studied fishing unit, with the ones in the special literature.

***Table 1***

**Normal growing limits in young rainbow trout**

| Age | R category | | I category | | II category | | III category | |
|---|---|---|---|---|---|---|---|---|
| | cm | g | cm | g | cm | g | cm | g |
| 1 months | - | - | 2,7-3,5 | 0,7 | 2-2,5 | 0,5 | 1,5-2 | 0,3 |
| 2 months | - | - | 5,5-6,5 | 1,5 | 2,5-5 | 1 | 3-3,5 | 0,8 |
| 4 months | - | - | 4,6-8,5 | 3,44 | 6,2-7 | 2,5 | 5-6 | 1,5 |
| 6 months | - | - | 12-14 | 18-20 | 8,1-10 | 13-15 | 7-8,1 | 6 |
| 10-12 months | - | - | 15-18 | 45,50 | 11-14 | 35-40 | 9-11 | 22-30 |
| 1,5 years | 20-22 | - | 20-22 | 80-100 | 18,20 | 60-80 | 16-18 | 40-60 |
| 2 years | - | - | 26-28 | 130-150 | 24,26 | 100-130 | 22-24 | 80-100 |
| 2,5 years | 28,29 | 200-220 | - | - | - | - | - | - |
| 3 years | 32-34 | 260-280 | - | - | - | - | - | - |

The table 2 presents the length growing results depending on age in young spring trout. It may notice that they have an increasing dynamics from 2, 29±0,191g at one month old to 13, 55±0,905 g at 1 year old, for the young spring trout livestock. Comparatively, the young rainbow trout recorded higher values even they started from the lower values, from 0, 50±0,051g at one month old to 38, 29±2,890 g at one year old, as it may be seen in table 3.

***Table 2***

**Length growing results depending on age in young spring trout**

| Age/weight | n | X±$s_x$ | S± | V% |
|---|---|---|---|---|
| 1 month | 30 | 2,29±0,191g | ±0,606 | 26,4 |
| 2 months | 30 | 4,50±0,382 g | ±1,208 | 26,8 |
| 4 months | 30 | 6,52±0,382 g | ±1,165 | 17,8 |
| 6 months | 30 | 9,53±0,726 g | ±2,296 | 24,6 |
| 12 months | 30 | 13,55±0,905g | ±2,862 | 21,1 |

***Table 3***

**Length growing results depending on age in young rainbow trout**

| Age/ weight | n | X±$s_x$ | S | V% |
|---|---|---|---|---|
| 1 month | 30 | 0,50±0,051g | ±0,163 | 32,6 |
| 2 months | 30 | 1,15±0,091g | ±0,287 | 25,0 |
| 4 months | 30 | 2,56±0,250 g | ±0,791 | 30,9 |
| 6 months | 30 | 12,34±1,588 g | ±5,018 | 40,67 |
| 12 months | 30 | 38,29±2,890 g | ±9,132 | 23,85 |

So, the results of the analyzed livestock recorded the individuals reared in Pucioasa fishing unit in the second quality category concerning their weight.

The table 4 presents the values of length and weight in 1, 5 years old rainbow trout. It may notice that they recorded a mean value of body length of 18,70±0,051cm, with a variability coefficient of 11,08%, that comparing with the standard values framed the analyzed livestock in the second quality category. Regarding the body weight values, the recorded mean value was 63,97±6,400 with a higher variability coefficient of 31,618%, and also included in the second quality category.

***Table 4***

**Length and weight growing results in 1, 5 years old rainbow trout**

| Parameter | n | X±$s_x$ | S | V% |
|---|---|---|---|---|
| Length cm | 30 | 18,70±0,051 | ±2,073 | 11,08 |
| Weight g | 30 | 63,97±6,400 | ±20,226 | 31,618 |

Table no 5 presents the mean results of length and weight growing in 2 years old rainbow trout. The mean value of body length was 26, 07±0,487 cm and the mean value of body weight was 128,37±6,171 g. Comparing these data with the ones in the special literature, it may conclude that for body length they are framed in the first quality category and for body weight in the second category.

*Table 5*

**Length and weight growing results in 2 years old rainbow trout**

| Parameter | n | $X \pm s_x$ | S | V% |
|---|---|---|---|---|
| Length cm | 30 | 26,07±0,487 | ±1,542 | 5,91 |
| Weight g | 30 | 128,37±6,171 | ±19,503 | 15,19 |

## 3. CONCLUSIONS

3.1. The recorded values regarding the length growing results depending on age in young rainbow and spring trout reveal the fact that even till 4 months old, the spring trout has higher values of body weight than rainbow trout, respective 6,52±0,382 g beside 2,56±0,250 g, after this age, the rainbow trout recorded higher values: 12,34±1,588 g beside 9,53±0,726 g in six months old and 38,29±2,890 g beside 13,55±0,905g.

3.2. Being assured a high food quality and best environmental conditions, the analyzed rainbow trout recorded at 1,5 years old 18,70±0,051 cm and 63,97±6,400 g.

3.3. The recorded data in the studied livestock of rainbow trout at two years old present a mean value of 26,07±0,487 cm body length and 128,37±6,171g.

3.4. Due to its fast growing rhythm rainbow trout has become the most preferred fish for the salmon breeders.

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# COMPARATIVE HISTOLOGICAL STUDIES OF THE SEMINAL LINE CELLS AT BOAR 10-70 DAYS

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Key words: seminiferous tubules, gonocytes, Sertoli cells, sustentacular cells, spermatogoniums

**SUMMARY**

In histological sections of testis taken from boars of 14-28 days, there is an increase in the average number of cells and gonocytes support. In this age period were not reported degeneration of gonocytes. There was an increase in the number of mitoses which included 4-6% of gonocytes examined.
At 42 days, apart from increasing the number of gonocytes into mitosis and cell growth appears degenerate. Seminiferous tubule lumen is almost shaped, are tunneled seminiferous tubules, and vasculature develops.
At the age of 70 days with gonocytes seminiferous tubules are present in the spermatogonial observed.

## 1. MATERIAL AND METHOD

The research has been conducted on a total of 104 testes collected from 52 different boars, of different breeds (Landrace, Duroc, Great White, Synthetic line Peris). Were aged between 10 and 70 days.

After histological processing was obtained numerous preparations were stained, examined and photographed with Nikon microscope.

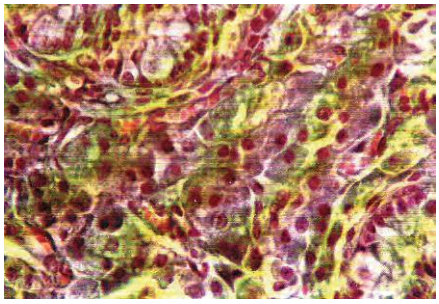
After examining and photographing the histological images were obtained which allowed studying seminal cell line.

## 2. REZULTS AND DISCUSSIONS

At the age of 10 days, the seminiferous tubules are small and disseminated in a mass of well developed connective tissue. Within the tubules were highlighted two types of cells: gonocytes and supporting cells.

In microscopic terms, in the prepubertal testicle peritubulare cells were found whit fibroblast-like appearance, and the seminal epithelium has one layer. Gonocytes have very large dimentions, globose nucleus

and the chromatin has a globular look. The Nucleus shows one or nucleoli. The arrangement may be basal or central. Supporting cells have a nucleus of small proportions, the chromatin is pulverulent and has granules located inside the nucleus.



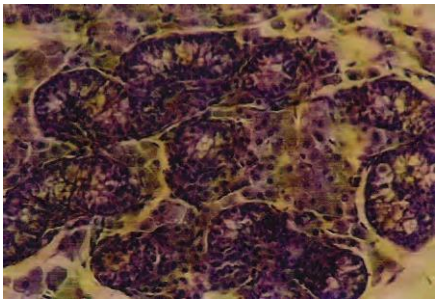


***Fig. 1 Boar testis by 10 days, Duroc breed. Fig. 2 Boar testis by 14 days, Great White breed***
**Seminiferous tube; Interstitial cells bounded by tubes grouped**
**Interstitial cells; Giemsa, x 200**
**Large cells with cytoplasmic strands; HE.,x200**

At this age the generations of the gonocytes which we suspect to be cells in apoptosis were observed in a small number of cells. The degenerative process was caracterissed thru the pyknotic aspect of the nucleus having clumps of chromatic cells in shape of islands. This clumps were not evenly spread and were usually located in front of the nuclear membrane. Degenerated cells had an hypertrophic and have a variable thickness membrane.

It clearly showed the characteristic structure of the limited membrane of the seminiferous tubules, the fine appearance and easily elastic of the al basal membrane also the presence of the conjunctive fibers arranged in concentric bundles concentrice specific to the fiber layer. In the mioid layer conjunctive cells are present having a endoteliform aspect and mioide cells . At the age of 21 days the tunneling process of the seminiferous tubules was started and the presence of cells in apoptosis can be noticed.

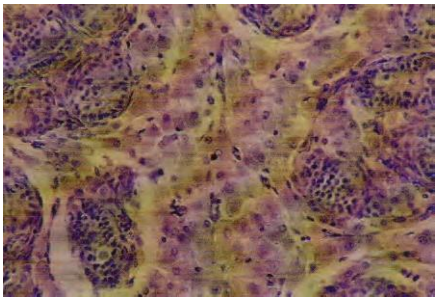


*Fig. 3 Boar testis by 21 days, Great White Peris breed.*
**Seminiferous tube;**
Interstitial cells; Giemsa x200

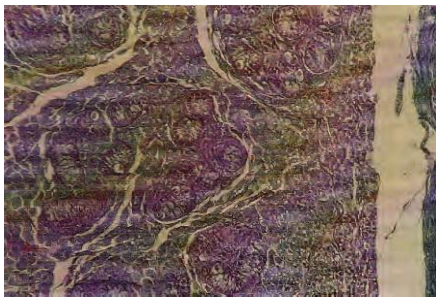


*Fig. 4 Boar testis by 21 days, Synthetic line* **Albuginea, seminiferous tubes netunnellisation.**
**Interstitial cells, apoptotic cells. HEA x 100**

The seminiferous tubules apear like some cords full of small cells, cubical or oval, grouped toward the center in a syncytial formation. At the age of 28 days the tunneling process of the seminiferous tubules is increasing, and in the Leydig cells cytoplasm is noticed the presence of vacuoule. Also at this age the interstitial cells appear richly vascularized.

At the age of 42 days the growth is directly proportional whit the average age number of support Sertoli cells and gonocytes .

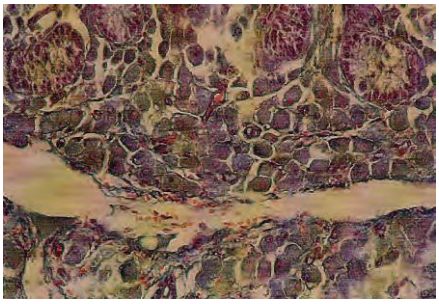


*Fig. 5 Boar testis by 28 days, Great White*

Interstitial glands well vascularized
HE x 200
Papenhain x 560

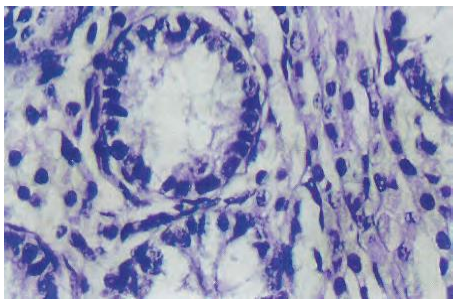


*Fig. 6 Testicle, bors species, breed Duroc, by 42 days*
Interstitial structure of the general tissue

Germinal epithelium monolayer is observed and some cells are in various stages of apoptosis.

At the age of 60 days the spermatogenesis process is starting it begins to appear in the epithelium of seminal primary spermatocytes. Also during this period is noticed a process of metaplasia of mesenchymal cells.

At the age of 70 days with gonocytes present seminiferous tubules is observed the spermatogoniums.

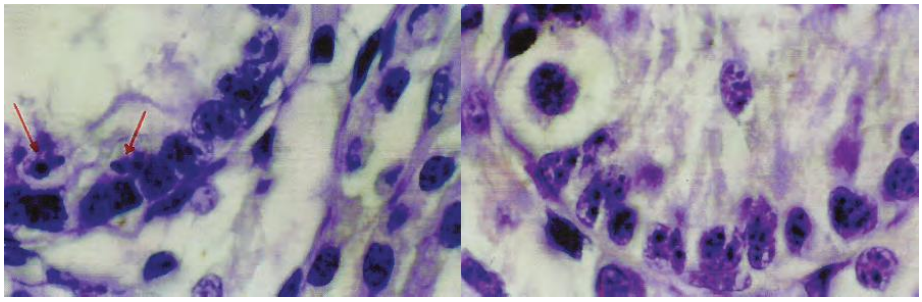


*Fig. 7 Testicle, boar species, breed Duroc, by 60 days.*
Process of metaplasia of mesenchymal cells

in the glandular cells and Leydig preglandular, adjacent of a seminiferous tube with spermatocite.
germinal epithelium monolayer.
Some cells are in various stages
(arrow)
Papenhain x 1400

*Fig. 8 boar species, Synthetic line Peris by 70 days.*
Germinal epithelium of seminiferous tubes with
predominantly cellular, spermatogoniums
with crust nucleus. Slowly primary

HE x 1400

## 3. CONCLUSIONS

3.1. At the age of 10 days, the seminiferous tubules are small and disseminated in a mass of connective tissue developed. Within the tubules were highlighted two types of cells and cells gonocytes support.

3.2. On histological sections of the harvested testis taken from the boars of 14-28 days, we notice a groth in the medium number of sustaining cells and of the gonocytes. In this age period were not noticed any degeneration of the gonocytes. A groth in number of the mitoses apeard witch contained 4-6% of the examind gonocytes.

3.3. At the age of 42 days, besides the numerical growth of the gonocytes in mitosis apears a growth of the degenerated cells. Seminiferous tubule lumen is almost shaped, are tunneled seminiferous tubules, and vasculature is intensifies.

3.4. At the age of 60 days the spermatogenesis process is starting it begins to appear in the epithelium of seminal primary spermatocytes.

Also during this period is noticed a process of metaplasia of mesenchymal cells.

3.5. At the age of 70 days with gonocytes present seminiferous tubules is observed the spermatogoniums.

## ACKNOWLEDGMENTS

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# MICROSCOPIC MORPHOLOGY OF THE SEMINAL LINE CELLS AT BOAR THE AGE OF 35 DAYS ON SMEARS AND TESTICULAR FINGERPRINT

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Key words: Key words: testicular fingerprint, smears, Leydig cells, spermatogonial dusty, spermatocytes

**SUMMARY**

Study of testicular imprints at 35 days stained by the method Toluidines Blanco revealed the presence of spermatogonial binucleate and multinucleated.
Type A spermatogonial dusty have nuclear chromatin with powdery appearance.
They divide mitotic and generate a spermatogonia by type A, which will continue to fulfill the role of stem cell for the line seminal and a spermatogonia intermediary.
The study of testicular imprints found this first-order spermatocytes and a spermatocytes by the second order.
The study of boar Leydig cells from testicular imprints multiple was observed the presence of nucleus multiple vacuolated in its peripheral area.
These cells appear polyhedral, irregular, nucleous are large, spheroidal, place eccentric with chromatin dense located peripheral, with nucleolus distincts, adjacent of nuclear membrane.
The study of testicular smeras using Bensley stain has revealed the presence of Leydig cells, some binucleate with nucleus polarized eccentric.

## 1. MATERIAL AND METHOD

The research has been conducted on a total of 30 testes collected from 15 different boars, of different breeds (Landrace, Duroc, Great White, Synthetic line Peris). Were aged 35 days.

The testicles were packed in gauze soaked with physiological saline, and ice around them. The operation of the sagittal section and removal of albuginea has made a fine shaving of the material after surface tissue section of the gonad with a very fine lamen.

Scrape material was diluted in a watch glass with saline at a rate by 1:1.

The mixture is then strip displays were put 2-3 drops of coloring to one extremity of the blades, and the dye penetrates through diffusion

For staining was used method Bensley modified.

Staining time is set to control the microscope objective 10 and eyepiece magnification with different power: 6; 10; 20; 40.

Smears thus obtained were examined under Nikon microscope by type Labophot 2 and then photographed.

Fingerprints testicular sagittal sectioning is achieved by the testis and a finally fat presses on the blade surface area of the gonad.

Colouring fingerprints testicular has made with 3-4 drops dye solution is placed over a slide. Stains are used Toluidin Blanco and Bensley modified.

Staining time is set to control the microscope, if the color has not released properly and cellular structures do not appear sufficiently colored dye solution is added to one end plate.

## 2. REZULTS AND DISCUSSIONS

They have a polyhedral shape, sometimes spheroid, the nucleus is big, eucromatic, nucleous have an eccentric position. The nucleus is dark, almost without structure and is int the center of the cell. Sometimes the nucleus is multiform being in division. After the appearance of the nucleus the three tipes of spermatogoniums can be noticed:

- dusty or A
- intermediate or I
- crusted or B

Spermatogonium dusty tipe A have the nuclear chromatin whit a pulverulent aspect.

They divide mitotic and generate a type A spermatogonium witch will continue to fulfillthe role of stem cell for the seminal line and a intermediary spermatogonia.

From the dusty spermatogoniums evolves as it is also visible in our testicular imprints preparations, the primary spermatocites. At this point it can be seen as dividing mitotic and will result a duty spermatogonia and a spermatocites I.

We can be observe the intermediate the spermatogoniums. They were oval, the nucleus takes the form of the cell being oval also, the chromatin is located peripheral and presents 2-3 nucleous. After dividing them crusted spermatogoniums of type B will result. These observable cells in preparations are small, have a spheroid shape. The Nucleus shape is oval, and the chromatin is crusted. Is observed that the mitotic division give rise to primary spermatocites.

The study of testicular imprints hawe shown the presence of spermatocites by primary and a spermatocites second order. Spermatocites by primary order are cells large (confirms the theory that are the biggest cells of the seminal line). They are spherical or oval and the nucleus has a reticular chromatin.

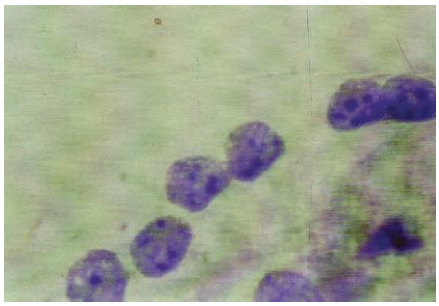

***Fig. 1 Testicular footprint boar species, Synthetic species, Landrce line Peris, by 35 days. Nucleos of spermatogoniums Toluidin Blanco x 1400 and spermatocites by Toluidin Blanco x 1400***

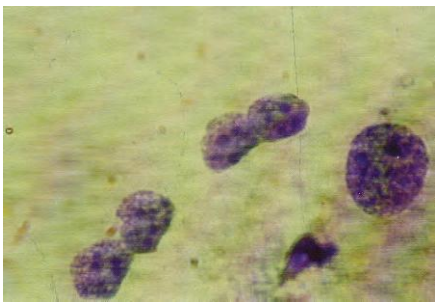

***Fig. 2 Testicular footprint boar by 35 days . Nucleos of spermatogoniums binucleolate and multinucleolate first degree.***

The cytoplasm is abundantly in mitochondria, containing numerous inclusions, Golgi complex is evident. Secondary spermatocytes by second orderar are small cells, 9-11μm in diameter, are haploid. They are formed by meiosis of primary spermatocytes. Have centrally located oval nucleus, chromatin is powdery. The cytoplasm is reduced, poorly basophilia.

The study of boar Leydig cells from testicular imprints multiple vacuolated nucleus was observed in the presence of its peripheral. In some preparations were observed giant Leydig cells, binucleate in peripheral area. Their study revealed the presence of early telophase during cell division. The nucleus appear visible binucleated. These cells appear polyhedral, irregular, the nucleous are large, spheroidal, eccentric place with dense peripheral chromatin located with distinct nucleous, adjacent nuclear membrane.

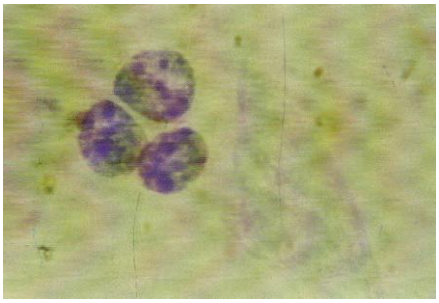

*Fig. 3 Testicular footprint boar species, Synthetic line Duroc by 35 days Spermatocites by first degree, with spheroidal nucleous and condensation nucleolar. Toluidin Blanco x 1400*

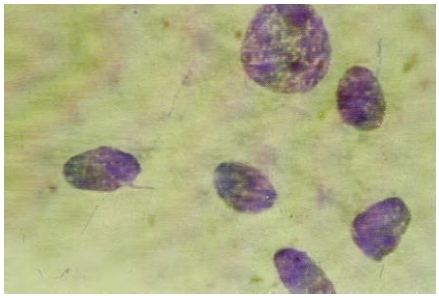

*Fig. 4 Testicular footprint boar species, Peris by 35 days Spermatogonius and a spermatocit with large nucleolus, eccentrically located Toluidin Blanco x 1400*

Nuclear membrane is irregular and shows pores. The cytoplasm is fibrillary through of abundance of agranular endoplasmic reticulum. Mitochondria are variable in size and shape and are more numerous in areas adjacent of agranular reticulum. In these areas accumulated fat droplets take the form of concentric membranes. Adjacent of nucleus concentric is observed endoplasmic reticulum concentric. A central portion of the cytoplasm near the nucleus of a pole have highly developed Golgi complex.

In addition, however, Leydig cells also presents lysosomes, granules, bundles of poliribosomi and changes in cell surface as: microcyli and vesicles can be seen pinocitosa phenomenon.

The study of smears testicular using Bensley stain has revealed the presence of Leydig cells, some binucleate with nucleous polarized, eccentric.

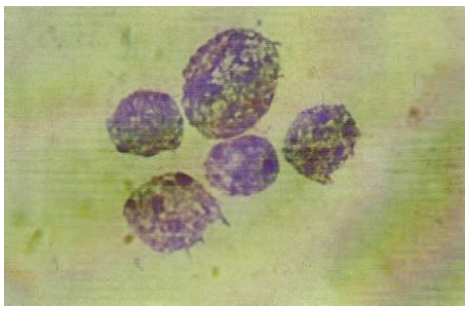

*Fig. 5 Testicular footprint boar species species*
*Great white by 35 days*
*Spermatocites by first and second degree degree*
*Nucleolate with a loose arrangement*
*Cromatinian. Toluidin Blanco x 1400*

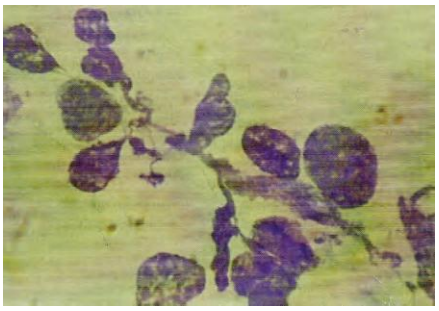

*Fig. 6 Testicular footprint boar species*
*Great white by 35 days*
*Spermatocites by first and second evolutionary line, arborescent type,*
*fine supporting connective.*
*Toluidin Blanco x 1400*

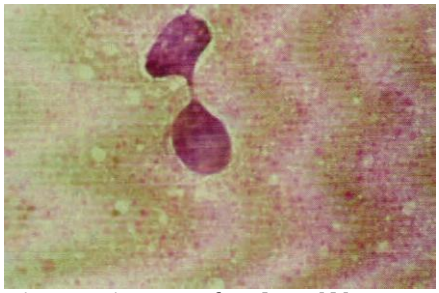

*Fig. 7 Testis smear of 35 days old boars, Syntetic line Peris. The divison of a spermatocites peripheral by first degrre. Bensely modified x 1400*

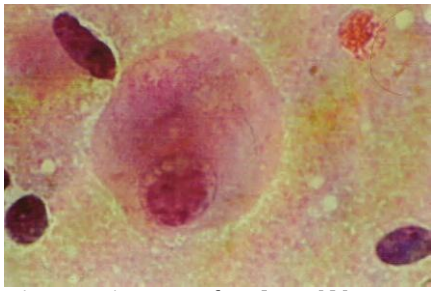

*Fig .8 Testis smear of 35 days old boars, Syntetic line Peris. Vacuolation coronary of nuclear mass of Leydig cells*
*Bensely modified x 1600*

## 3. CONCLUSIONS

3.1. The Study of testicular imprints at the age of 35 days stained using the method Toluidin Blanco have showmen the presence of spermatogoniums binucleate and multinucleated.

3.2. The nucleus appear visible binucleated. These cells appear polyhedral, irregular, the nucleous are large, spheroidal, eccentric place with dense peripheral chromatin located with distinct nucleous, adjacent nuclear membrane.

3.3. The cytoplasm is fibrillary through of abundance of agranular endoplasmic reticulum. Mitochondria are variable in size and shape and

are more numerous in areas adjacent of agranular reticulum. In these areas accumulated fat droplets take the form of concentric membranes.

3.4. Adjacent of nucleus concentric is observed endoplasmic reticulum concentric. A central portion of the cytoplasm near the nucleus of a pole have highly developed Golgi complex.

## ACKNOWLEDGMENTS

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# HISTOPLASMOSIS WITH ATYPICAL LOCATION OF THE DOG, IN TIMIŞOARA CITY - CASE STUDY

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**Key words**: histoplasmosis, immunodeficiency, dog, Timisoara

**SUMMARY**

This article describes a case of disseminated histoplasmosis in a dog, 9 months, German Shepherd, who suffered from a severe chronic colitis, refractory to treatment with antibiotics, metronidazole respectively, examined at a veterinary surgery in Timisoara. Laboratory evaluation of blood showed anemia, decreased hemoglobin and decreased PCV
Examination of blood smear showed marked lymphopenia and neutrophilia. Further investigation revealed the presence of specific yeast cells histoplasmei both direct smears made from rectal mucosa, respectively in culture. The infection was most likely orally, because the clinical level of distress indicate a digestive system.

Histoplasmosis is a disease caused by a dimorphic and diphazic fungus a pathogen morphotype yeast present in the soil, *Histoplasma capsulatum* (*H. capsulatum*), saprophytic, causing infections, but nonpathogenic, with good adaptability to the host (1, 2, 4, 7).

Although these are described systemic mycosis in humans and most mammals, especially in endemic areas, there is no evidence of direct transmission from animals to humans, although it occurs in animals and humans shared the same environment (2, 3, 5). The disease can be asymptomatic or may develop a benign development, but in rare cases can progress and clinical condition may be serious and even fatal. Original location, is characteristic of the lung, similar to tuberculosis, often accompanied by mediastinitis and an exuberant fibrotic response. The spread of this infection may be due to concomitant development of other diseases that alter the immune balance. Regarding clinical aspects, there are two forms of the disease, benign and inaparentă, which is characterized by pulmonary nodules, and a fatal form of affecting the pulmonary reticuloendothelial system, namely an atypical enteric form (2, 4, 6, 7).

## 1. MATERIAL AND METHODS

In May 2009, at a veterinary clinic in Timisora, went to counseling a 9-month dog German shepherd breed, with symptoms of enteritis, with fluid faecal, smelly, brown, with chronic evolution. Overall health was not affected initially, later in the disease, appeared apathy, anorexia and weight loss. All these symptoms made him the owner to seek veterinary medical services. Duration of development until submitted to the cabinet was 3 weeks. During this time he benefited from veterinary advice, treatment with antibiotics (Synulox tabl., 12.5 mg/kg/12 hours, per os) for 14 days without any obvious clinical remediation. After clinical evaluation, and blood samples were collected urine, respectively, to assess Bioclinica the medical analysis laboratory in Timisoara.

The results of hematologic laboratory examination revealed anemia (RBC 4.06 x 1012 L), with values of hemoglobin (7.9 g/L) and low PCV (25.4%), while blood smear examination showed lymphopenia (5%) and neutrophilia (20% of nonsegmented neutrophil granulocytes were represented). Liver enzymes after laboratory analysis (ALT/AST) showed no significant changes.

Parameters results from the analysis of urine samples from dogs examined were within the reference values, except for a moderate proteinourie.

To set up a hydro rebalancing treatment (Duphalyte - 50ml/kg, Hartman infusion, infusion of 5% glucose), together with antimicrobial therapy, enrofloxacin (15mg/kg/12 hours), the systemic route.

After one week, the diarrhea persisted, leading only to improve the general condition. On veterinary advice, the case was routed to the Veterinary Medicine University Clinics Timisoara.

Using pharyngeal exudate collected tubes without culture medium was collected from the rectum, by scraping, rectal mucosa, which was subsequently submitted to an examination bacterioscopic respectively mycological on selective media for mycetes, Sabouraud dextrose, with added antibiotics (Gentamicin 10 000 μg/ml) and agar with 5% calf blood, incubated at 25 ° C, respectively at 37 ° C aerobically for six days.

## 2. RESULTS AND DISCUSSION

After incubating on Sabouraud dextrose agar resulting on surface granular colonies, white-woolly appearance, which became brown with time, while blood agar grown colonies were creamy.

Issues resulting from the development of microscopic slides from different colonies were obtained as follows: septated hyphae, smooth or wart macroconidii wall and were unicellular microconidiile nonpigmentate (if developed colonies on agar Sabouraud dextrose) and burjeonate yeast cells (in smears obtained colonies on blood agar).

In direct smears made from the intestinal mucosa prelevatele stained May Grunvald Giemsa was observed stronger infestation with histoplasma in macrophages (Fig. 1).

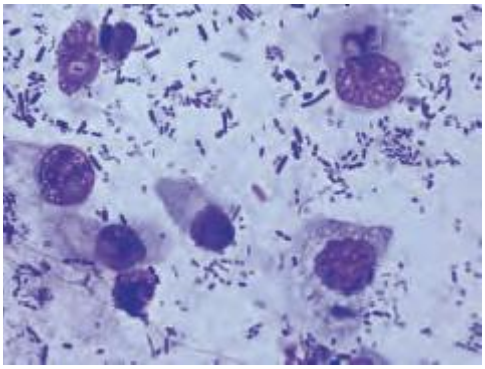

**Fig. 1. Scraper rectal mucosa of dog. Combination of individual epithelial cells and macrophages loaded with bacteria from a mixed background. Levuriforme cells are present in macrophages (May Grunvald Giemsa, 100X).**

*The diagnosis* was based on results of cytological examination and culture examination. After this stage, *specific treatment* was instituted using: ketokonazol (Nizoral tabl. 100 mg) dose of 10 mg/kg/12 hours for 3 weeks, associated with a liver protective herbal LIV 52 (human use), 2 tabl./day orally throughout the period of antifungal therapy.

At the end of treatment were harvested from new rectal mucosa samples for mycological examination. It was negative and the animals clinical condition markedly improved, the signs entirely digestive disease have disappeared.

## 3. CONCLUSIONS

3.1. Epidemiological data have reported that the dog is walked twice daily in a park on land where many droppings of wild birds (*Corvus frugileus*);

3.2. *H. capsulatum*, a exosaprobiot living, usually outdoors, where it feeds on dead organic matter, but in certain circumstances, may enter the host organism to become pathogenic;

3.3. The transition from saprophytic to the parasitic life is done under natural conditions, inhalation of chlamydospores microlevuri and that will give rise levuriforme cells to host body;

3.4. Histoplasma proliferate quickly and easily avoiding the mononuclear phagocyte system of the host immune system. This being the examined disease case epidemiological chain.

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# RESEARCHES REGARDING THE INCIDENCY OF INFESTATION WITH *OTODECTES CYNOTIS* IN CATS

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**Key words:** cats, *Otodectes cynotis*, otitis externa

**SUMMARY**

Occasionally, cats can be the source of many diseases in their living environment. They can transmit disease directly to other animals and on humans. These may include important parasitic disease caused by ectoparasites, which in many cases can be some unusual or zoonotic risk. Evolving periodic ectoparasites, may be an important source for transmission of diseases and hypersensitivity phenomena may trigger skin diseases in cats and, especially, can cause serious anemia in young animals with long term treatment. The most common example is the ear mite, *Otodectes cynotis*, which colonizes the external ear canal and outer ear in dogs and cats. In his opinion Sotiraki et al. (13), *Otodectes cynotis* is responsible for at least half of feline diseases worldwide. Mehlhorn (8), believes that 80% of cats that wander in Europe are carriers of this parasite. Cats and foxes are among the species commonly *Otodectes cynotis* carrier, transmitting the parasite in dogs and humans.

## 1. MATERIAL AND METHODS

The objectives of this study are to determine the degree of infestation of external ear the cat with *Otodectes cynotis* mite.

The research was conducted in 2005-2007 in Timisoara and Arad city, a total of 298 cats, belonging to different races (European, British shorthair, Persian, Burmese, Siamese, Russian Blue and Norwegian Forest) aged 3 months to 14 years. Cats were initially subjected to a thorough clinical examination, which included the dermatological examination and otoscope examination. Following these tests, of all cats examined were diagnosed with otitis externa (OE), a total of 58 cats.

Otitis externa is one of the reasons (rare) for the cats come to counseling, compared with dogs. However, this condition may represent more than 25% of cases consulted to diagnose general skin problems and 2% of dermatological cases sent for consultation (6, 8, 11, 13).

To determine the etiology of this pathological entity, were harvested from the depths of that flag hearing and ear canal of both ears,

pathological material, represented by skin scrape and otic exudate. In this respect were harvested using sterile tubes, which were subsequently processed in the laboratory for etiologic diagnosis. For parasitological examination, stick with otic exudate was treated immediately with 20% glycerinate KOH solution and subjected to the fight laborious microscopic examination. Mite identification was based on morphological aspects.

## 2. RESULTS AND DISCUSSION

Of cats diagnosed with OE, *O. cynotis* positive were 25 cats, representing 43.1% of the cases affected by ear infections. Were diagnosed 7 cats with severe clinical signs of OE, which in addition to the mites have been isolated and many Gram positive and negative bacteria.

Cats in this category have their habitat both in home and outside it. Clinical signs observed were: ear scratching, head shaking, redness of the internal surface of the auricular pavilion, associated with variable amounts of ear discharge, dark brown and gritty appearance. These are aspects resemble typical clinical OE cases associated with *O. cynotis*.

During this study, cats with OE were monitored in terms presence of infestation with *O. cynotis* to one ear (left or right), or both ears. The results obtained on this parmeter showed right ear infestations presence where 32% of cats diagnosed with parasitic OE, respectively 24% of cats, the left ear. Mites were identified in both ears, with a frequency of 44% (Table 1 and Fig. 1).

***Table 1.***

**Frequency of cats infected with Otodectes cynotis.**

| Number and frequency (%)infected ears | | | |
|---|---|---|---|
| Left ear | Right ear | Both ears | Total cats |
| 6 (24%) | 8 (32%) | 11 (44%) | 25 (100%) |

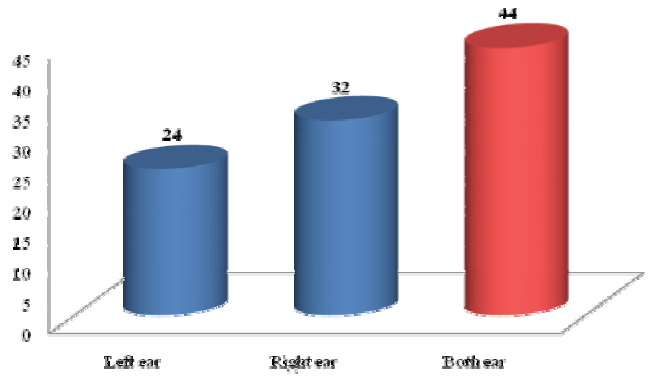


**Fig. 1. Locating infestation with *O. cynotis* to cat (%)**

Among the clinical signs that betray the presence of infestations with mites, level of external ear canal the cats, is itching, respectively oto-podal reflex. The findings were similar to data reported and described in the literature (4, 5, 12).

Thus all 25 cats diagnosed with parasitic OE, caused by *O. cynotis*, showed pruritus (Table 2). The presence of itching in the ear, could be associated with the presence of mites in the ear canal of cats.

***Table 2.***

**The number of cats examined for diagnosis infestation with *O. cynotis.***

| *Otodectes cynotis* | Presence of itching | | |
|---|---|---|---|
| | Pozitive | Negative | Total |
| **Pozitive** | 25 | - | 25 |
| **Negative** | 14 | 19 | 33 |
| **Total** | 39 | 19 | 58 |

Also in this study were monitored and factors associated with infestation with *O. cynotis* the cat, so assessing their role in triggering and maintaining infestations. Study of the factors include: age, sex, race, hair length and type, the animals living environment, ear hygiene frequency and not the least contact with other animals (Table 3 and Fig. 2).

*Table 3.*

**Factors associated infestation with *Otodectes cynotis* to cat.**

| Variable factors | | No. of animals examined with OE | Infestation with *Otodectes cynotis* | |
|---|---|---|---|---|
| | | | No. of animals examined (%) | |
| **Age group** | Youth | 23 | 18 | 72 |
| | Adults | 20 | 5 | 20 |
| | Old | 15 | 2 | 8 |
| **Sex** | Male | 30 | 14 | 56 |
| | Females | 28 | 11 | 44 |
| **Race** | Pure race | 21 | 4 | 16 |
| | European | 37 | 21 | 84 |
| **Hair length** | Short | 28 | 21 | 84 |
| | Long | 12 | 3 | 12 |
| | Medium | 18 | 1 | 4 |
| **Type of hair** | Smooth | 29 | 6 | 24 |
| | Coarse | 16 | 16 | 64 |
| | Irregular | 13 | 3 | 12 |
| **Living environment** | Outside the house | 24 | 21 | 84 |
| | Inside the house | 34 | 4 | 16 |
| **The frequency of ear hygiene** | Once a week | 46 | 25 | 100 |
| | Three times a week | 8 | - | - |
| | Daily | 4 | - | - |
| **Contact with other animals** | Cats | 29 | 14 | 56 |
| | Cats and dogs | 21 | 11 | 44 |
| | Without contact | 8 | - | - |

When referring to the sex of affected animals, no significant differences were found between males (56%) and females (44%) (Table 3 and Fig. 2). Age, as seen from the results of the study, young animals are most affected (72%) compared with other age groups. It may be a factor in the infestation with *O. cynotis* (Fig. 2). If we refer to breed affected animals, we observed that European breeds of cats are much more affected (84%) than those of pure breeds (16%) (Fig. 2).

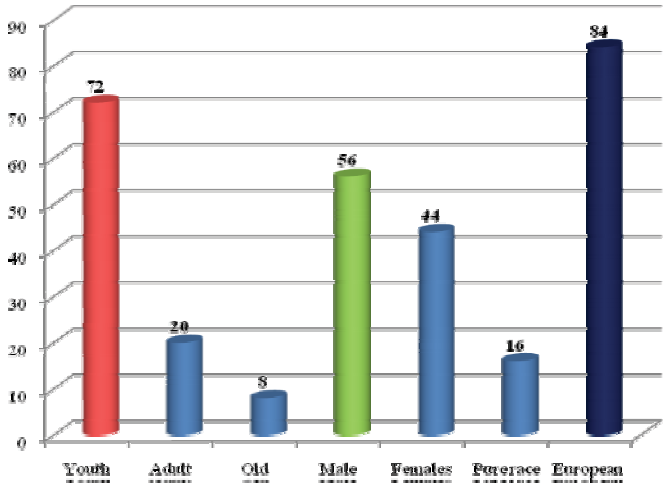


**Fig. 2. Evolution of factors related to age, gender and breed in infestation with *O. cynotis* to cat (%).**

The length and type of hair, affecting only to a certain extent infestation with these mites. Cats with short hair (84%) were most affected, namely those with coarse (64%), followed at a distance the outstanding smooth haired (24%).

This may be associated with the fact that cats most common breed (European), have this type of hair growth, and that can put the account number of cases with parasitic OE diagnosed in this race (Fig. 2).

After analyzing the data, the cats living environment is another important factor, both in starting and maintaining infestations in cats. Thus, animals kept outside the home are more prone to infestation with *O. cynotis*.

The degree of infestation was 84% among cats diagnosed with parasitic OE. Cats reared in the home, or in an isolated environment from the outside, it seems that they are much less likely to be infected. In the study conducted, these cats have been diagnosed only 16% of the total affected cats (Fig. 3)

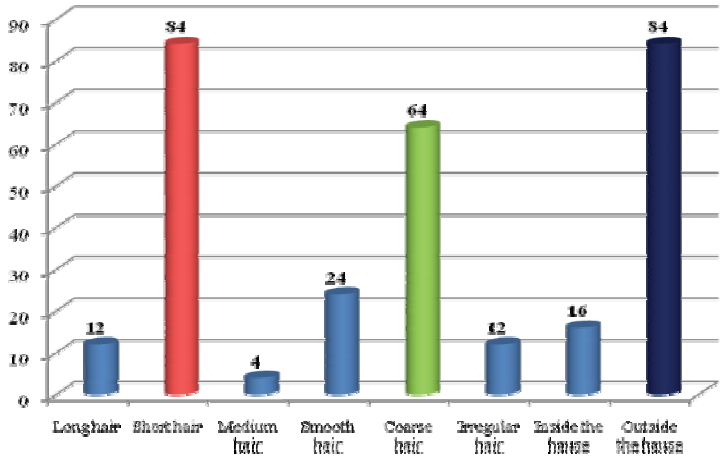


**Fig. 3. Evolution of factors related to length and type of hair, and living environment in infestation with *O. cynotis* to cat (%).**

Among the factors studied, was also related to the frequency of ear hygiene and contact with other animals that either the species or other species. It is known that as ear cleaning is done more frequently, the possibilities of developing an ear diseases are lower, and the diagnosis of a possible problem occurs more rapidly, helping to establish a timely therapy, reducing possibilities of complications with various infections.

All cats have been diagnosed as parasitic OE caused by *O. cynotis* were most frequently cleaned in ear once a week (Fig. 4). Another factor described, was the contact with other animals. By following it, we could demonstrate their role in the transmission and spread of infestation with *O. cynotis* in a population of cats. Most cats become infected have had contact with other cats (56%), and a certain part and dogs (44%). There were no reported infestations in cats which had contact with any animal (Fig. 4).

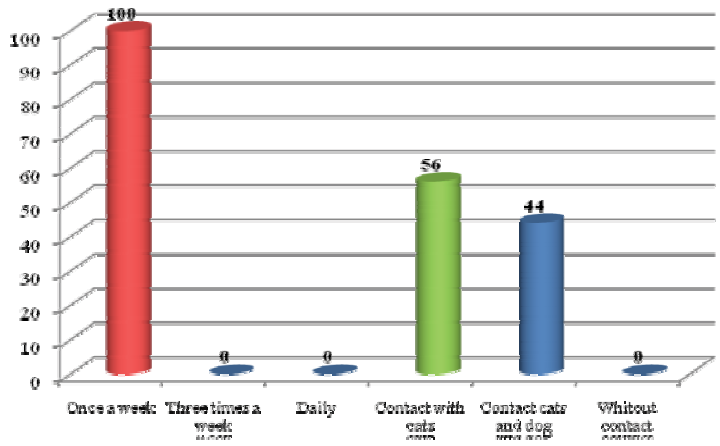


**Fig. 4. Evolution of factors related to frequency ear cleaning, and contact with other animals in infestation with *O. cynotis* to cat (%).**

Ear mite is a commonly parasite present in all cats throughout the world, their prevalence is between 5-50%, depending on several factors. Age, growth and maintenance conditions of animals, habitat social and immunological status (development of a hypersensitivity) may be plausible explanations.

If superinfection is possible that the examination result to be negative, because, due to unfavorable environmental mites migrated extraauricular.

The differential diagnosis must be made to the other ear ceruminous and/or itching (seborrheic otitis, otitis with *Malassezia spp.* with *Culicoides stomoxys* ear dermatitis, Notoedric scabies, mange scabies, trombiculosis).

Two other studies conducted in the USA, reports a low prevalence of infestation with ear mites in cats between 5-7% (McCallum 1967 and Murphy et al. 1982). Compared with these results, the rate of infestation can be much larger population of cats studied, but is low due to an erroneous reports (Gram et al. 1994).

In Greece Sotiraki et al. (13), in a study of 161 domestic cats, reported 25.5% of the animals studied were infested with *O. cynotis*.

In Florida, Akucewich et al. 2002, describing a prevalence of infestation with these mites by 37%.

Ito Naoyuki and Ito Sayako in 2002, investigated the prevalence of *Otodectes cynotis* mite on 679 domestic cats, taking into account the living environment, age, national origin, sex, race and examined season.

Mite was detected in 64 cats (9.4%). Prevalence of infestation has been reported in cats kept outside the home (16.6%), compared with those in the house (8.3%), or those kept in both ways (4.6%). No significant differences were described in terms of age, sex, origin, race and season examined (3).

Rataj et al. (10) examined the period 1998-1999, the bodies of 101 cats from Ljubljana to detect ectoparasites in the ear. The cats were transported to the laboratory of pathology, and have undergone a full examination to determine cause of death among other animals. Using a swab was collected from the depth nvelul flag that ff the external ear canal secretions and wax, which was immediately treated with 10% KOH. From currently no study reported that 34.65% of the cats were infested with ectoparasites, such *Otodectes cynotis* with 32.7%, with *Felicola subrostratus* (4.95%) and how *Notoedres cati* (1.98%).

## 3. CONCLUSIONS

Synthetic analysis, the cases considered in this comparative study a detachment following conclusions:

3.1. No significant differences were found between tomcats and cats that are affected by parasitic otitis caused by *O. cynotis* (male 14/30 – 56%; female 11/28 – 44%). Young animals are more affected than other age groups (18/23 – 72%). European breed cats are much more affected than those of pure breeds (21/37 – 84%);

3.2. Living environment of cats is an important factor in triggering and maintaining infestations. Animals kept outside the home, are more prone to infestation with *O. cynotis* than those reared in the home (21/24 – 84%);

3.3. Frequent cleaning of the ear, reduce the likelihood of infestations, as well as complicated cases. Most infected cats have had contact with other cats, respectively dogs (14/29 – 56%, respectively 11/21 – 44%). There were no reported infestations in cats which had contact with any animal;

3.4. The length and type of hair, only slightly affect infestation with these mites. Cats with short hair were the most affected, namely those with coarse, followed at a distance from the hair smooth (21/28 – 84%; 64% and 6/29 – 24%). This may be associated with the fact that most cats of European race, has this type of hair growth, and that may be made on account of the large number of cases with parasitic OE diagnosed in cats of this breed.

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# RESEARCH REGARDING THE DISTRIBUTION OF MANDIBULAR BRANCH OF THE TRIGEMINAL NERVE IN SMALL RUMINANTS

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**Key words:** trigeminal nerve, maseterin, distribution, ruminants

**SUMMARY**

Because the mandibular branch of trigeminal nerve has a complex distribution and integrates both motor and sensitive nerves, with many individual variations, we conducted research on 10 goats and 10 sheep, pointing the most important aspects. As described, the features regarding the posting of the deep temporal maseterin nerve and those on the path of buccinator nerve. There were differences on the morphology of otic ganglion, not described in the literature yet. We met variants of superficial temporal nerve distribution and we have described in detail the two motor branches which have intimate relationship with the mandibular branch.

It at humans, knowledge regarding the cervico-cefalic branches vascularization, innervations and conformation are well known, at domestic animals, the mentioned field was relatively scarcely approached. There are studies regarding the somatic intervention of the cephalic zone at small ruminals, but they are limited either at sheep (May, 1970; Nicolescu *et al*, 1969; Defenzy Sodja, 1984), either at goats (Predoi *et.al*, 2001) and contain few comparative data. There were studied in comparison the ophthalmic and maxilla branch of the trigeminal (Godinho şi Getty, 1973), reason for which in this present work we studied three mandibular branch.

## 1. MATERIALS AND METHODS

The study material was represented by ten goat heads and ten ovine heads. Animals destined for dissection, demonstration and reasearch in the Domestic Animal Anatomy Laboratory from The Faculty of Veterinary Medicine were used. The method used was disection, executed bilateral, to the visibility limit, using the SMZ-2T Nikon stereo microscope. Nerves and colagene fibers were brushed with acetic acid or with methylene blue so thay can be differentiate.

## 2. RESULTS AND DISCUSSIONS

The mandible branch of the trigeminal nerve leaves the cranial cavity through the oval whole and after a short path it emits a series of branches which have certain features (Fig.1)

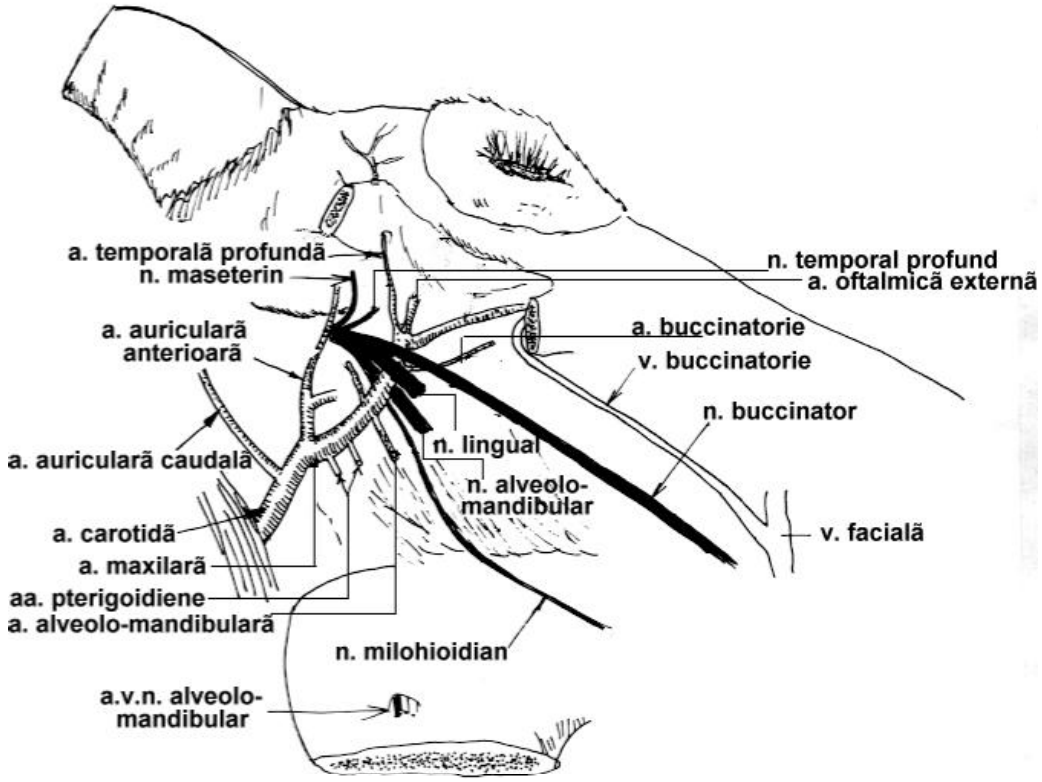


**Fig. 1 The mandibular branch of the trigeminal nerve at goats (schemă).**

The masterin nerve appears relatively shorter and thicker. Before penetrateing the sigmoid notch it emits two deep temporal branches, which, in 80% of the cases, can fusion. After passing the notch it splits in two separate branches, especially at goats: a thinner one for the superficial and middle lay-out of the masseter muscle, and a thicker one for the deeper lay-out.

The buccinator nerve, isolated from the mandibular nerve at the same level with the previous, has a special path. After it crosses the medial pterigoidian muscle in rostro-ventral direction, it reaches on the lateral side of the molar section of the buccinator muscle, appearing almost superficial at the anterior edge of the maseter muscle. It goes down through the superior and middle molar glands, then it goes in the mass of the inferior molar glands where it is lost in the cheek mucous membrane.

On it's path, close to is's origin, it emits, most of the time, two muscles for the temporal muscle at sheep and just one branch at goats and branches for the lateral pterygoid muscle. The otic ganglion, that the literature show's it quite developed, has appeared, in most of the cases, extremily reduced.

The superficial temporal nerve, unlike what can be found at equines, is very thin, but contrary to those affirmed in literature, it is not always simple. In over 40% of the cases we found it duble, and in some cases even triple. Yet, it is true that, if multiple branches exist, they appear as ramifications of the common trunk. The superficial temporal nerve at sheep constantly issues an anastomosis branch with the auriculo palpebral nerve. Instead the superficial temporal nerve contribution at forming the sub zygomatic plex is much lower than at any other species.

The lingual nerve is extremely bulky, and it is joining with coarda timpanului nerve near from its origin , the last being very short.

The milo-hioidian nerve appears thicker than at other species.

The alveolo mandibular nerve penetrates through the mandibular whole in the inferior dentar duct.

The existence of a thin nervous branch issued by the mandibular nerve at the interosseous elbow is observed, branch that comes out through a small special channel that openes at the back of the last molar. That nerve is casted, on one side to the last inferior molar, on the other side to the posterior pillar of the tongue.

## 3.CONCLUSIONS

3.1. The maseterin nerve appears relatively short and thick. Before it reaches in the sygmoid notch it emits two profound temporal branches, which in 80% of the cases can fusion.

3.2. The buccinator nerve issues threads for the temporal nerve, two at sheep and just one at goats.

3.3. The otic ganglion, which the literature presents it quite developed, appeared extremely reduced in most of the cases;

3.4. The superficial temporal nerve, unlike what can be observed at equines, is very thin, but contrary to that affirmed by the literature, it is not always simple. In over 40% of the cases we found it double and in some of the cases even triple.

3.5. The lingual and the milohioidian nerve are highly represented.

3.6. The alveolar mandibular nerve presents a special branch which comes out a thin channel that opens at the back of the last molar. That nerve is casted, on one side to the last inferior molar, on the other side to the posterior pillar of the tongue.

## ACKNOWLEDGEMENTS.

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# THE EFFECT OF SOME POLYPHENOLIC EXTRACTS UPON OXIDATIVE STRESS IN RATS WITH ASCITOGENOUS HEPATIC TUMORS

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**Key words**: polyphenols, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thiobarbituric acid reactive substances (TBARS).

## SUMMARY

In this paper it is reported the effect of *Vaccinium myrtillus, Hypericum perforatum* and *Chelidonium majus* extracts (100 mg polyphenols / kg−1 body weight) on the antioxidant profile of rat liver. Activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were increased and reduced lipid peroxidation in liver homogenates. Administration of plant polyphenols to normal rats decreased TBARS levels to 90.9% (for normal control rats treated with bilberry extract) and 81.8% (for normal control rats treated with St. John's Wort extract), compared to normal rats group. The level of oxidative stress can enhance SOD activities in cancer: +40.9% for implanted tumor rats treated with bilberry extract. The CAT activities were higher (by 41.6 %, 57.7 % and 52.9 % respectively) compared to control rats with implanted tumors. The administration of plant extracts to rats with cancer resulted in highly significant increase in GPx activities, as compared to normal control rats, $p < 0.01$.

Reactive oxygen species (ROS) occur both during normal metabolic reactions and following exposures to various factors, such as smoking, atmospheric pollutants, UV light, ionizing radiation, and xenobiotics. The extracellular environment and cells have different antioxidant systems, including enzymatic and non-enzymatic antioxidant molecules. However, several enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) etc. act as antioxidants to influence oxidative stress. SOD catalyzes the dismutation of $O_2$•- into hydrogen peroxide ($H_2O_2$), which can be transformed into water and molecular oxygen by CAT. GPx also reduces $H_2O_2$, as well as lipid hydroperoxides, and may therefore help to prevent damage from lipid peroxidation. Polymorphisms in these enzymes are supposed to be associated with DNA damage and subsequently the individual's risk of cancer susceptibility. While some reports have noted increased enzymatic antioxidant activities in rats and mice induced with carcinogens (Manju and Nalini, 2005), others have reported decreased or no changes of enzymatic antioxidant status in carcinogenesis induced

animals (Chandra Mohan and Nagini, 2003; Karimov *et al.*, 2003). Since tumor promotion is closely related to oxidative stress, a compound that exhibits antioxidative properties is expected to act as an antitumor promoter. Antioxidative capacities of *Vaccinium myrtillus, Hypericum perforatum* and *Chelidonium majus* increased enzymes activity. Plant extracts were able to attenuate the harmful effects of tumors by scavenging the increased radicals generated during hepatocarcinogenesis. Flavonoids have been found to play important roles in the non-enzymatic protection against oxidative stress, especially in case of cancer (Babich *et al.*, 2005; Okada *et al.*, 2001).

## 1. MATERIALS AND METHODS

***Preparation of ethanolic extracts***

In this study there were used dried aerial parts of bilberry *(Vaccinium myrtillus),* St. John's wort *(Hypericum perforatum)* and greater celandine *(Chelidonium majus).* The interest parts of plants were powdered and extracted with ethanol 60 % (1:10 ratio, w:v) for 3 hours at 60ºC. The homogenates obtained were filtered using filter paper Watman no. 1 and the filtrates were then centrifuged for 20 min at 5000 rpm and 5ºC. After the ethanol was evaporated, the aqueous residues were utilized.

***Animals***

Forty adult female albino rats, weighing 150-170 g were used as experimental animals in this study. They were kept in cages under standard laboratory conditions of 12 h/12 h light/dark, 25 ± 2°C with free access to food and water. All the pharmacological experimental protocols respected European legislation for experimental animals.

***Experimental protocol***

The rats were randomly divided into 8 groups of 5 animals each, as follows:

- Group 1: Normal control rats treated with 0.9% NaCl: rats were orally administered 1 mL 0.9% NaCl, for 10 days.
- Group 2: Normal control rats treated with bilberry extract: rats were orally administered polyphenols in a dose of 100 mg kg−1, for 10 days.
- Group 3: Normal control rats treated with St. John's wort extract: rats were orally administered polyphenols in a dose of 100 mg kg−1, for 10 days.
- Group 4: Normal control rats treated with greater celandine extract: rats were orally administered polyphenols in a dose of

100 mg kg−1, for 10 days.

- Group 5: Control rats with implanted tumors treated with 0.9% NaCl: rats were orally administered 1 mL 0.9% NaCl, for 10 days.
- Group 6: Rats with implanted ascitogenous hepatic tumors treated with bilberry extract: rats were orally administered polyphenols in a dose of 100 mg kg−1, for 10 days.
- Group 7: Rats with implanted ascitogenous hepatic tumors treated with St. John's Wort extract: rats were orally administered polyphenols in a dose of 100 mg kg−1, for 10 days.
- Group 8: Rats with implanted ascitogenous hepatic tumors treated with greater celandine extract: rats were orally administered polyphenols in a dose of 100 mg kg−1, for 10 days.

Twenty-four hours following last administration, the animals were sacrificed by cervical dislocation. The abdomen was excised and the liver was removed immediately by dissection, washed in ice-cold isotonic saline and blotted between two filter papers. The liver was transferred into preweighed vials to determine the wet weight. A 10% (w/v) liver homogenates was prepared in ice-cold 0.1 M potassium phosphate buffer, pH 7.5.

***Determination of lipid peroxidation***

The measurement of liver lipid peroxide by a colorimetric reaction with thiobarbituric acid was done as described by Ohkawa et al. (Ohkawa *et al.*, 1979), and the determined lipid peroxide is referred to as malondialdehyde. Briefly, in a test tube, 20% trichloroacetic acid solution and 0.67% thiobarbituric acid solution were added to the homogenate. The color of thiobarbituric acid pigment was developed in a water bath at 100°C for 20 min. After cooling with tap water to room temperature, 2mL *n*-butanol was added and shaken vigorously. After centrifugation, the color of butanol layer was measured at $\lambda_{max}$ 532 nm. The TBARS concentration of the sample was calculated using the extinction coefficient of MDA ($1.56 \times 10^5$ $M^{-1}cm^{-1}$) and the values were expressed as nmol/mg protein.

***Determination of superoxide dismutase activity (SOD)***

The activity of superoxide dismutase in liver was measured using a commercial kit (Fluka analytical). This method uses xanthine and xanthine oxidase to generate superoxide radicals which react with 2- (4-iodophenyl)- 3- (4- nitrophenol)- 5- (2, 4- disulfophenyl)-2H-tetrazolium, monosodium salt to form a water soluble formazan dye. The values are expressed as Units/mg of protein in liver tissue.

***Determination of catalase activity (CAT)***

Catalase activity was measured by the method described by Aebi (Aebi, 1984). Supernatant was added to cuvette containing 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of freshly prepared 30 mM $H_2O_2$. The rate of decomposition of $H_2O_2$ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as Units/mg of protein.

***Determination of glutathione peroxidase activity (GPx)***

The method is based on the oxidation of gluthatione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (Nicotinamide Adenine Dinucleotide Phosphate, Reduced) (Gupta and Baquer, 1998). Final reaction mixture (3 ml) was 65 mM $KH_2PO_4/K_2HPO_4$, pH 7.5, 2 mM GSH, 1 U glutathione reductase, 0.12 mM NADPH and 8 mM tert buthyl hydroperoxide. Activity of glutathione peroxidase was expressed as Units/mg of protein.

***Determination of total proteins***

Total proteins were determined according to the method of Lowry, using bovin seric albumin (BSA) as a standard (Lowry *et al.*, 1951).

***Statistical data interpretation***

Statistical data interpretations were calculated with EXCEL program from Microsoft Office package. All the data are shown as mean value ± standard deviation (SD). Number of rats per group n = 5. Statistical data interpretation considered the corresponding differences for a given significance threshold: $p>0.05$ statistically insignificant; $*p<0.05$ statistically significant; $**p<0.01$ strong statistical significance; $***p<0.001$ very strong statistical significance.

## 2. RESULTS AND DISCUSSIONS

***Determination of lipid peroxidation***

The level of TBARS is an indicative for lipid peroxidation in hepatic cells. Administration of plant polyphenols to normal rats decreased TBARS levels by 9.1% (for normal control rats treated with bilberry extract) to 18.2% (normal control rats treated with St. John's wort extract), compared to the normal rats (Table 1).

*Table 1*

**The influence of oral administration of plant polyphenols (100 mg/kg) to rats on antioxidant enzymes activity [superoxide dismutase (SOD); catalase (CAT) and glutathione peroxidase (GPx)]**

| Animal Groups | TBARS nmol/mg protein | SOD U/mg protein | CAT U/mg protein | GPx U/mg protein |
|---|---|---|---|---|
| **Group 1** | 2.2 ± 0.6 | 61.4 ± 1.87 | 49.6 ± 2.6 | 16.3 ± 1.3 |
| **Group 2**<br>**% Change from group 1** | 2± 0.13<br>- 9.1% | 66.5 ± 2.13<br>+ 8.3 % | 51.6 ± 2.7<br>+4.0 % | 16.9 ± 1.4<br>+ 3.7 % |
| **Group 3**<br>**% Change from group 1** | 1.8 ± 0.8<br>- 18.2% | 68.8 ± 2.01<br>+ 12.1 % | 54.3 ± 1.5<br>+ 9.5 % | 16.8 ± 1.2<br>+ 3.1 % |
| **Group 4**<br>**% Change from group 1** | 1.9 ± 0.8<br>- 13.6% | 64.7 ± 1.30<br>+ 5.4 % | 53.7 ± 2.4<br>+ 8.3 % | 17.1 ± 0.9<br>+ 4.9 % |
| **Group 5**<br>**% Change from group 1** | 18.5 ± 0.8<br>+ 740.9 % | 42.3 ± 1.52<br>- 31.1 % | 29.3 ± 1.8<br>- 40.9 % | 10.5 ± 1.2<br>- 35.6 % |
| **Group 6**<br>**% Change from group 5** | 15.2 ± 0.8<br>- 17.8 % | 59.6 ± 1.99<br>+ 40.9 % | 41.5 ± 1.9<br>+ 41.6 % | 14.3 ± 0.7<br>+ 36.2 % |
| **Group 7**<br>**% Change from group 5** | 11.3 ± 0.5<br>- 38.9% | 53.9 ± 1.37<br>+ 27.4 % | 46.2 ± 2.1<br>+ 57.7 % | 13.9 ± 0.9<br>+ 32.4 % |
| **Group 8**<br>**% Change from group 5** | 14.4 ± 0.9<br>- 22.2 % | 61.2 ± 1.86<br>+ 44.7 % | 44.8 ± 2.2<br>+ 52.9 % | 15.1 ± 0.5<br>+ 43.8 % |

TBARS level showed a huge significant increase by +740.9% in rats with implanted tumors in comparison to the normal control group. Moreover, polyphenols supplementation to implanted tumors rats improved the level of TBARS, compared to implanted liver tumors control rats. Implanted tumors elevated TBARS from 2.2 ± 0.6 nmol/mg protein to 18.5 ± 0.8 nmol/mg protein. This value was decreased to 15.2 ± 0.8 nmol/mg protein, 11.3 ± 0.5 nmol/mg protein and 14.4 ± 0.9 nmol/mg protein in case of groups treated with bilberry, St. John's Wort and greater celandine.

***Determination of superoxide dismutase activity (SOD)***

Treatment of normal rats with plant extracts showed an increase of SOD activity. The decrease in SOD activity of tumor implanted rats liver is obvious: 61.4 ± 1.87 U/mg protein (normal rats) to 42.3 ± 1.52 U/mg protein (tumor-implanted rats). The levels of SOD activities in liver homogenates were significantly improved upon treatment of tumor implanted rats with 100 mg/kg plant polyphenols which inhibited hepatic injuries induced by cancer. Thereby, the level of oxidative stress

can enhance antioxidant enzyme activities in cancer: +40.9% for tumor implanted rats treated with bilberry extract, +27.4% for tumor implanted rats treated with St. John's wort extract and +44.7% for tumor implanted rats treated with greater celandine extract (Fig. 1).

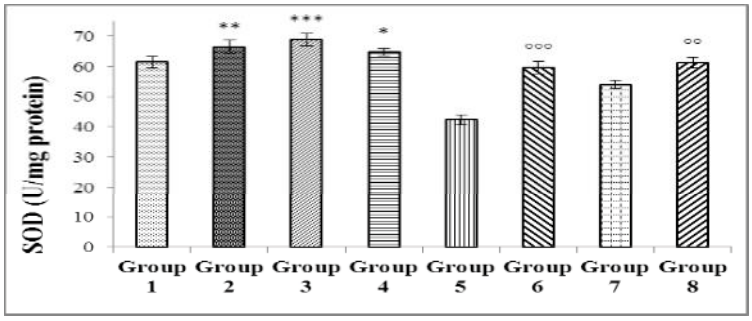


**Fig. 1. The influence of oral administration of plant polyphenols (100 mg/kg) on SOD activities in the liver of normal and tumor implanted rats. Data are expressed as mean ± S.D. Number of rats per group n = 5. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs 1st rats group. °° $p < 0.01$ vs 4th rats group °°° $p < 0.001$ vs 2nd rats group.**

### *Determination of catalase activity (CAT)*

Catalase is a ubiquitous antioxidant enzyme which catalyses the decomposition of hydrogen peroxide ($H_2O_2$) to water and oxygen. Hydrogen peroxide is formed in the eukaryotic cell as a by-product of various oxidases and superoxide dismutases. Hydrogen peroxide accumulation in cells causes oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death. Removal of the $H_2O_2$ from the cell by catalase provides protection against oxidative damage to living cells and its role in oxidative stress related diseases has been widely studied.

Highly significant reduction in CAT status was observed in rats with cancer (Group 5) when compared with control group rats: -40.9 % (Table 1). These adverse changes were reversed to near normalcy and an improvement in antioxidant status was noticed for 6th, 7th and 8th groups (Fig. 2).

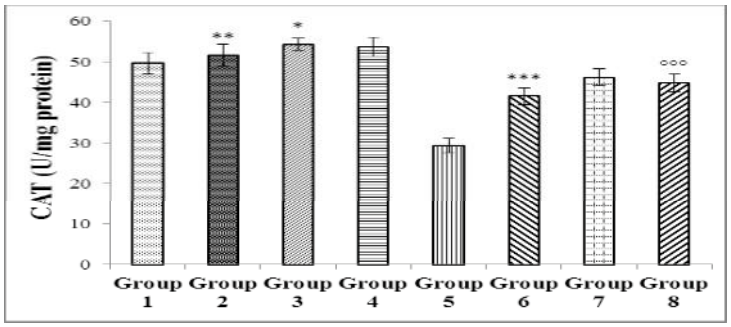


**Fig. 2. The influence of oral administration of plant polyphenols (100 mg/kg) on CAT activities in the liver of normal and tumor implanted rats. * $p < 0.05$, ** $p < 0.01$ vs 1st rats group. *** $p < 0.001$ vs 2nd rats group. °°° $p < 0.001$ vs 4th rats group**

### *Determination of glutathione peroxidase activity (GPx)*

The decreased GPx level of tumor implanted rats leads to an increase of toxic level to the cells. The decreased activities of GPx and SOD in liver homogenate of tumor implanted rats may be due to oxidative stress that induces enzymes inactivation (Ostrowska *et al.*, 2004). Also, it was reported that decreased GPx activity leads to $H_2O_2$ accumulation in the liver which in turns inactivates SOD (Kakkar *et al.*, 1997).

The GPx activities in the liver homogenates markedly decreased in rats with cancer (Group 5) when compared with control group rats: -35.6% (Table 1). In liver homogenates from 6th, 7th and 8th groups, the plant extracts prevented the loss of GPx activities and showed significant values ($p < 0.01$) comparable to liver homogenates obtained from 2nd, 3rd and 4th groups values (Fig. 3).

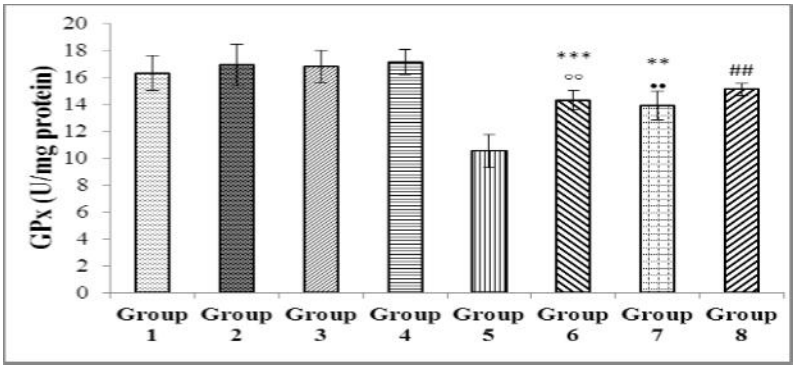


**Fig. 3. The influence of oral administration of plant polyphenols (100 mg/kg) on GPx activities in the liver of normal and tumor implanted rats. *** $p < 0.001$ vs 5th rats group. °° $p < 0.01$ vs 2nd rats group.** $p < 0.01$ vs 5th rats group. •• $p < 0.01$ vs 3rd rats group. ## $p < 0.01$ vs 4th rats group**

## 3. CONCLUSIONS

3.1. Polyphenols supplementation to tumors implanted rats groups improved the level of TBARS, compared to control rats with implanted tumors.

3.2. The treatment of tumor implanted rats with plant polyphenols (100 mg/kg) significantly improved the levels of SOD activities in liver homogenates.

3.3. Polyphenols extracted from bilberry *(Vaccinium myrtillus),* St. John's wort *(Hypericum perforatum)* and greater celandine *(Chelidonium majus)* improved the levels of CAT activity in liver homogenates.

3.4. The GPx activities in liver homogenates obtained from tumor implanted rats groups treated with plant extracts were almost similar to normal control rats group.


## ACKNOWLEDGEMENTS

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# EFFECTS OF VARIOUS PLANT POLYPHENOLS ON LIPID PEROXIDATION, REDUCED GLUTATHIONE AND SOME ENDOGENOUS ENZYMES IN STRESSED MICE

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**Key words**: plant polyphenols, antioxidant enzymes, glutathione (GSH), thiobarbituric acid reactive substances (TBARS).

## SUMMARY

The aim of the study was to investigate the effect of hawthorn *(Crataegus monogyna),* wild pansy *(Viola tricolor)* and nettle *(Urtica dioica)* extracts on the occurrence of oxidative stress in the liver of mice by measuring the extent of oxidative damage as well as the status of the antioxidant defense system. Plant polyphenols were administered orally (100 mg/kg body weight) and the levels of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and reduced glutathione (GSH) were estimated in mice with induced oxidative stress. The levels of TBARS were increased in stressed control mice by +149.02 % in comparing to the normal control group. Administration of plant extracts to stressed mice decreased the levels of lipid peroxidation. The decrease in SOD activity of the stressed mice liver was evident: 9.2 ± 0.8 U/mg protein (normal mice) to 4.8 ± 0.7 U/ mg protein (stressed mice). Plant polyphenols administration on stressed mice showed significant increase in CAT status: 1.8 ± 0.3 U/mg protein for hawthorn *(Crataegus monogyna),* 2.1 ± 0.3 U/mg protein for wild pansy *(Viola tricolor)* and 2.5 ± 0.4 U/mg protein for nettle *(Urtica dioica)*, while CAT level in stressed mice was 1.4 ± 0.2 U/mg protein. Plant polyphenols administration improved GST activities in oxidative stress induced mice: 5.4 ± 0.3 U/mg protein for hawthorn *(Crataegus monogyna),* 6.1 ± 0.4 U/mg protein for wild pansy *(Viola tricolor)* and 6.8 ± 0.5 U/mg protein for nettle *(Urtica dioica).* The activities of GSH were decreased in stressed mice: 18.6 ± 2.3 mg/ 100 g tissue when compared to normal mice group: 36.7 ± 2.2 mg/ 100 g tissue.

Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, hydrogen peroxide are produced in living systems under normal conditions and the body handles free radicals formed by scavenging them through the endogenous enzymatic (SOD, GPx, GST, CAT) and non enzymatic antioxidants (glutathione, bilirubin, vitamins). However, enhanced concentrations of ROS and circulating lipid peroxidation product (MDA) have been implicated with carcinogenesis (Chung *et al.*, 2001; Manju and Nalini, 2005). Based on the available evidence, living organisms do not only require endogenous protective antioxidants to scavenge free radicals, but they also require exogenous antioxidants such as vitamins A, C, E, flavonoids and polyphenols derived from plants. Plant foods might have critically

important components for oxidative prevention because of their capacity to scavenge free radicals (Lambert *et al.*, 2005).

## 1. MATERIALS AND METHODS

### Preparation of ethanolic extracts

In this study, there were used dried aerial parts of hawthorn (*Crataegus monogyna*), wild pansy (*Viola tricolor*) and nettle (*Urtica dioica*). The interest parts of plants were powdered and extracted with ethanol 60 % (1:10 ratio, w:v) for 3 hours at 60ºC. The homogenates obtained were filtered using Watman no. 1 filter paper and the filtrates were then centrifuged for 20 min at 5000 rpm and 5ºC. The extracts were used after ethanol was evaporated.

### Animals

The animals used in this study were purchased from Cantacuzino Institute. In this study, forty adult female albino mice (25-30 g weight, 9 weeks old) were used as experimental animals. They were kept in polypropylene cages under standard laboratory conditions of 12 h/12 h light/dark, 22 ± 2°C temperature, fed with a normal rodent diet one week before the experiment. Starvation was used prior to all assays because polyphenols extracts were always administered orally (by gavage) using distillated water as vehicle. Oxidative stress was induced in experimental albino mice by keeping them in special lighting conditions (6 hours of daylight and 18 hours of darkness). The administration of plant extracts to mice began seven days before inducing oxidative stress. All the pharmacological experimental protocols respected European legislation for experimental animals.

### Experimental protocol

The mice were randomly divided into eight groups of five animals:

- Group 1: Normal control mice treated with distillated water.
- Group 2: Normal control mice treated with hawthorn extract: mice were orally administered polyphenols in a dose of 100 mg $kg^{-1}$, for 21 days.
- Group 3: Normal control mice treated with wild pansy extract: mice were orally administered polyphenols in a dose of 100 mg $kg^{-1}$, for 21 days.
- Group 4: Normal control mice treated with nettle extract: mice were orally administered polyphenols in a dose of 100 mg kg−1, for 21 days.

- Group 5: Control mice with induced oxidative stress: mice were treated with distillated water, for 21 days.
- Group 6: Mice with induced oxidative stress treated with hawthorn extract: mice were orally administered polyphenols in a dose of 100 mg kg−1, for 21 days.
- Group 7: Mice with induced oxidative stress treated with wild pansy extract: mice were orally administered polyphenols in a dose of 100 mg kg−1, for 21 days.
- Group 8: Mice with induced oxidative stress treated with nettle extract: mice were orally administered polyphenols in a dose of 100 mg kg−1, for 21 days.

Twenty-four hours following last administration, the animals were sacrificed by cervical dislocation. The abdomen was excised and the liver was removed immediately by dissection, washed in ice-cold isotonic saline and blotted between two filter papers. The liver was transferred into preweighed vials to determine the wet weight. 10% (w/v) liver homogenates were prepared in ice-cold 0.1 M potassium phosphate buffer, pH 7.5.

***Determination of lipid peroxidation***

The measurement of liver lipid peroxide by a colorimetric reaction with thiobarbituric acid was done as described by Ohkawa et al. (Ohkawa H. *et al.*, 1979). Briefly, in a test tube, 20% trichloroacetic acid solution and 0.67% thiobarbituric acid solution were added to the homogenate, in a final volume of 2 mL. The color of thiobarbituric acid pigment was developed in a water bath at 100°C for 20 min. After cooling with tap water to room temperature, 2 mL *n*-butanol was added and shaken vigorously. After centrifugation, the color of butanol layer was measured at $\lambda_{max}$ 532 nm. The TBARS concentration of the sample was calculated using the extinction coefficient of MDA ($1.56 \times 10^5$ $M^{-1}cm^{-1}$) and the values were expressed as nmol/mg protein.

***Determination of superoxide dismutase activity (SOD)***

The activity of superoxide dismutase in liver was measured using a comercial kit (Fluka analytical). This method uses xanthine and xanthine oxidase to generate superoxide radicals which react with 2- (4- iodophenyl)- 3- (4- nitrophenol)- 5- (2, 4- disulfophenyl)-2H- tetrazolium, monosodium salt to form a water soluble formazan dye. The values are expressed as Units/mg of protein in liver tissue.

***Determination of catalase activity (CAT)***

Catalase activity was measured by the method described by Aebi (Aebi, 1984). Supernatant (0.1 mL) was added to cuvette containing 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of

freshly prepared 30 mM $H_2O_2$, in a final volume of 3 mL. The rate of decomposition of $H_2O_2$ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as Units/mg of protein.

***Determination of glutathione peroxidase activity (GPx)***

The method is based on the oxidation of gluthatione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (Nicotinamide Adenine Dinucleotide Phosphate, Reduced) (Gupta and Baquer, 1998). GPX activities were measured by linking the reaction to that of glutathione reductase and following the decrease in NADPH at 340 nm (extinction coefficient of $6.22\times10^3$ $M^{-1}\times cm^{-1}$) at 25°C, for 5 min. Activity of glutathione peroxidase was expressed as Units/mg of protein.

***Determination of liver glutathione S-transferase (GST) activity***

Estimation of the liver GST activity was carried out by the method of Habig *et al.* (Habig *et al.*, 1974). The reaction mixture consisted of 1.425 mL 0.1M phosphate buffer (pH 6.5), 0.2 mL of 1 mM reduced glutathione, 0.025 ml 1mM 1-chloro-2,4-dinitrobenzene (CDNB) and 0.3 ml 10% liver homogenate in a total volume of 2.0 ml. The changes in absorbance were recorded at 340 nm and enzymatic activity was calculated as nmol CDNB conjugate formed / min / mg protein using a molar extinction coefficient of $9.6 \times 10^3$ $M^{-1}$ $cm^{-1}$.

***Determination of liver reduced glutathione (GSH)***

Reduced glutathione in liver was determined by the method of Jollow *et al.* (Jollow *et al.*, 1974). An aliquot of liver homogenate (10% in 0.1M phosphate buffer) was precipitated with sulfosalicylic acid (4%). The samples were kept at 4°C for 1h and then subjected to centrifugation at 4000 rpm for 15 min at 4°C. The assay mixture contained 0.1 mL aliquot from the supernatant, 0.1M phosphate buffer (pH 7.4) and dithionitrobenzene (DTNB) in a total volume of 3.0 ml. The optical density of the yellow color developed was read immediately at 412 nm in a spectrophotometer. GSH was expressed as mg / 100 g tissue using a GSH standard curve.

***Determination of total proteins***

Total protein was determined according to the method of Lowry *et al.* using bovine seric albumin (BSA) as a standard (Lowry *et al.*, 1951).

Statistical data interpretation

Statistical data interpretation was calculated with EXCEL program from Microsoft Office package. Statistical data interpretation considered

the corresponding differences for a given significance threshold: $p>0.05$ statistically insignificant; $*p<0.05$ statistically significant; $**p<0.01$ strong statistical significance; $***p<0.001$ very strong statistical significance.

## 2. RESULTS AND DISCUSSION

### *Determination of lipid peroxidation*

The level of TBARS is an indicative for lipid peroxidation in hepatic cells. Fig. 1 illustrates lipid peroxidation values in liver of normal and experimental mice.

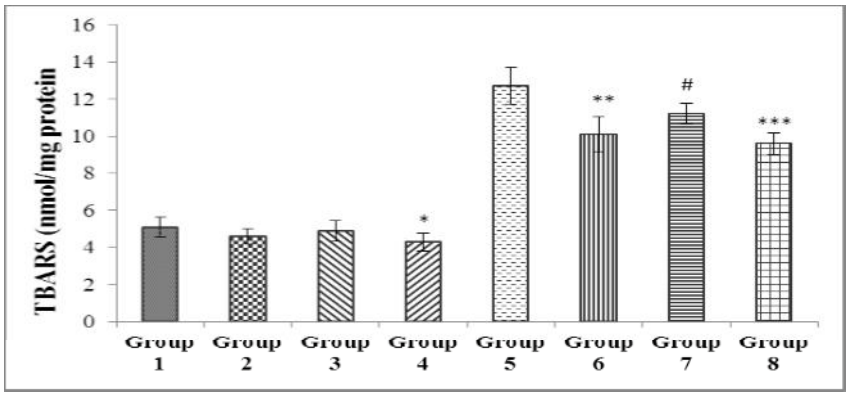


**Fig. 1. The influence of oral administration of plant polyphenols (100 mg/kg) on lipid peroxidation in the liver of normal and stressed mice. Data are expressed as mean ± S.D. Number of mice per group n = 5. * $p < 0.05$ vs 1st mice group; ** $p < 0.01$, *** $p < 0.001$ and # $p < 0.05$ vs 5th mice group.**

TBARS levels were increased in stressed control mice by +149.02% comparing to the normal control group. Administration of plant extracts to stressed mice decreased lipid peroxidation. Treatment of normal mice with plant extract showed significant changes in lipid peroxidation (for nettle ($p<0.05$)). The effect produced by plant extracts administration on stressed mice presented biological significance when compared with stressed untreated group. The influence of oral polyphenols administration (100 mg/kg) on lipid peroxidation in the liver of normal and stressed mice was determinated as 5.1 ± 0.5 nmol/mg protein for Group 1; 4.6 ± 0.4 nmol/mg protein for Group 2; 4.9 ± 0.6 nmol/mg protein for Group 3; 4.3 ± 0.5 nmol/mg protein for Group 4; 12.7 ± 1.0 nmol/mg protein for Group 5; 10.1 ± 0.9 nmol/mg protein for Group 6; 11.2 ± 0.5 nmol/mg protein for Group 7; 9.6 ± 0.6 nmol/mg protein for Group 8 (Fig. 1).

***Determination of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities***

Treatment of normal mice with plant extracts showed an increase of SOD and CAT activity when compared with stressed untreated group. The decrease in SOD activity of the stressed mice liver is evident: 9.2 ± 0.8 U/mg protein (normal mice) to 4.8 ± 0.7 U/ mg protein (stressed mice). The levels of SOD activity in liver homogenates were significantly improved after the treatment of stressed mice with 100 mg/kg plant polyphenols: 5.8 ± 0.3 U/mg protein for hawthorn, 5.3 ± 0.4 U/mg protein for wild pansy and 6.6 ± 0.7 U/mg protein for nettle (Table 1). Highly significant reduction in CAT status was observed in stressed untreated mice (Group 5) when compared with control group mice; the decrease was from 4.2 ± 0.5 U/mg protein to 1.4 ± 0.2 U/mg protein (Table 1). Plant polyphenols administration on stressed mice showed significant increase in CAT status: 1.8 ± 0.3 U/mg protein for hawthorn, 2.1 ± 0.3 U/mg protein for wild pansy and 2.5 ± 0.4 U/mg protein for nettle. The CAT level in control stressed mice was 1.4 ± 0.2 U/mg protein.

***Table 1***

**The influence of oral administration of plant polyphenols (100 mg/kg) to mice on antioxidant enzyme activities [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST)].**

| Animal Groups | SOD U/mg protein | CAT U/mg protein | GPx U/mg protein | GST U/mg protein |
|---|---|---|---|---|
| **Group 1** | 9.2 ± 0.8 | 4.2 ± 0.5 | 5.1 ± 0.5 | 8.4 ± 0.6 |
| **Group 2** | 9.3 ± 1.1 | 4.3 ± 0.5 | 5.4 ± 0.4 | 8.5 ± 0.5 |
| **Group 3** | 9.1 ± 0.9 | 4.4 ± 0.6 | 5.3 ± 0.4 | 8.6 ± 0.5 |
| **Group 4** | 9.3 ± 0.6 | 4.2 ± 0.5 | 5.2 ± 0.3 | 8.7 ± 0.3 |
| **Group 5** | 4.8 ± 0.7 | 1.4 ± 0.2 | 1.8 ± 0.4 | 2.9 ± 0.2 |
| **Group 6** | 5.8 ± 0.3 | 1.8 ± 0.3 | 3.2 ± 0.4 | 5.4 ± 0.3 |
| **Group 7** | 5.3 ± 0.4 | 2.1 ± 0.3 | 2.9 ± 0.4 | 6.1 ± 0.4 |
| **Group 8** | 6.6 ± 0.7 | 2.5 ± 0.4 | 4.1 ± 0.4 | 6.8 ± 0.5 |

The GPx activities in the liver homogenates markedly decreased in untreated stressed mice (1.8 ± 0.4 U/mg protein), when compared with control group mice (5.1 ± 0.5 U/mg protein) (Table 1). In liver

homogenates from groups 6 and 7, plant extracts increased GPx activities and showed significant values ($p < 0.01$ for $7^{th}$ group and $p < 0.001$ for $8^{th}$ group) compared to $5^{th}$ group values (Table 1).

In addition to SOD, CAT and GPx, there are numerous antioxidant enzymes reacting with and detoxifying compounds produced by oxidative stress (Sies, 1993). Glutathione S-transferase catalyses the conjugation of GSH to various endogenous and exogenous electrophilic compounds (Halliwell and Gutteridge, 2007). The depletion of GSH content may also lower the GST activity (Halliwell and Gutteridge, 2007).

The activities of GST were decreased in stressed mice (2.9 ± 0.2 U/mg protein) when compared to normal mice group (8.4 ± 0.6 U/mg protein). Plant polyphenols administration improved GST activities in oxidative stress induced mice: 5.4 ± 0.3 U/mg protein for hawthorn, 6.1 ± 0,4 U/mg protein for wild pansy and 6.8 ± 0.5 U/mg protein for nettle.

***Determination of liver reduced glutathione (GSH)***

Under *in vivo* conditions, GSH acts as an antioxidant and its decrease was reported in oxidative stress (Halliwell and Gutteridge, 2007). The activities of GSH were decreased in stressed mice (18.6 ± 2.3 mg/ 100 g tissue) when compared to normal mice group (36.7 ± 2.2 mg/ 100 g tissue) (Fig. 2). Plant polyphenols administration improved GSH levels in oxidative stress induced mice: 27.5 ± 2.3 mg/100 g tissue for hawthorn, 30.3 ± 2.5 mg/100 g tissue for wild pansy and 29.5 ± 3.1 mg/100 g tissue for nettle.

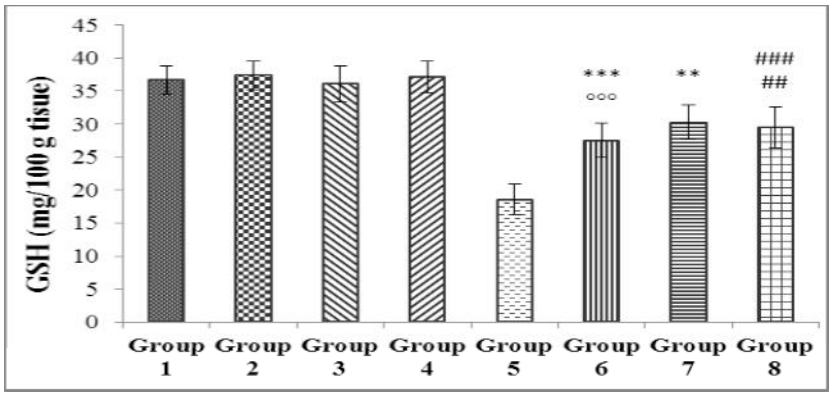


**Fig. 2. The influence of oral administration of plant polyphenols (100 mg/kg) on GSH levels in the liver of normal and stressed mice. Data are expressed as mean ± S.D. Number of mice per group n = 5. *** $p < 0.001$ and ### $p < 0.001$ vs $5^{th}$ mice group. °°°$p < 0.001$ vs $2^{nd}$ mice group.** $p < 0.01$ vs $3^{rd}$ mice group. ## $p < 0.01$ vs $4^{th}$ mice group.**

## 3. CONCLUSIONS

3.5. Polyphenols supplementation to stressed mice decreased the level of TBARS, compared to stressed control mice.

3.6. The treatment of stressed mice with plant polyphenols in quantity of 100 mg/kg significantly improved the levels of SOD, CAT, GPx and GST activities in liver homogenates.

3.7. Polyphenols extracted from hawthorn *(Crataegus monogyna),* wild pansy *(Viola tricolor)* and nettle *(Urtica dioica)* improved the levels of GSH in stressed mice liver homogenates when compared with stressed untreated mice.

## ACKNOWLEDGEMENTS

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# CASE STUDY – ENCEPHALITIS IN A 5 MONTHS OLD BOXER

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**Key words:** dog, encephalitis, neurology

**SUMMARY**

In this paper we are discussing the case of a 5 months old female boxer dog, which first arrived in our clinic feeling sick and showing neurological signs. After taking the case history, the clinical, neurological and complementary exams, the diagnosis given was that of encephalitis and the appropriate treatment was started (the treatment was adapted from human medicine).

A 5 months old dog was brought into our practice after feeling sick for a couple of days at home. The dog was walking with its head down, drifting to the left side and its front legs spread widely. The dog had received treatment at another practice for Babesia and the diagnosis that we established was that of encephalitis due to the infestation with the parasite.

## 1. MATERIALS AND METHODS

The case was studied and treated at the Medical Clinic of the FMV, Bucharest; the clinical and neurological exams done in the Medical Clinics and for a certain diagnosis we sent the patient for a MRI exam at Colentina Hospital.

For this case, the steps in diagnosis and treatment were as following:

Case history
Clinical exam
Blood exam and biochemistry exam
Serum exam (Carré disease, Babesiosis)
Eye exam
Radiologic exam
Neurologic exam

## 2. RESULTS AND DISCUSSIONS

The patient arrived in our clinic at the end of June 2010 after a weekend during which it felt worse and worse.

The dog was up to date with its vaccination status; internal and external parasite control was done less than a month before.

After a detailed history was taken the clinical examination (including the neurological testing) revealed the following:

Apathy, depression

The dog seemed disorientated

Ataxia of the forelimbs;

During walking the animal held its head down and was drifting to the left;

The spinal reflexes were normal for all limbs;

The cranial reflexes were either delayed or absent; the right eye had permanent strabismus.

The panicullus and the perineal reflexes were normal.

The submandibular and popliteal lymph nodes were larger than normal, on both sides.

The patient was receiving treatment at another practice for Babesia infestation, she had received the antidote (Imizol) and the signs had appeared after this.

After the clinical examination we decided to undergo the following:

Hematology exam:

Normal values

Biochemistry exam:

No detectable changes

Urine analysis:

Normal values

Carré disease test:

Negative

Borelia test:

Negative

Babesia test

Negative

MRI exam was not done due to financial reasons

The treatment underwent was as such:

Omega-3 – 500mg once a day after a meal; 90 days

Betaserc – 12 mg/day, after a meal; 30 days;

Cerebrolysin – 100mg/day, after a meal; 10 days

Clyndamicin – 10mg/kg/day, after a meal; 20 days

We recommended that the owner use a product for preventing external parasite infestation on the dog.

The patient came back after 20 days for a follow-up, her general status was very good, and the neurological signs were all gone.

The dog will come for check-ups on a regular basis.

## 3. CONCLUSIONS

When examining a case with undefined symptoms we must establish if it is indeed a neurological case or not.

The case history, the clinical exam and the neurological exam are as important as the laboratory tests.

The differential diagnosis in all tick spread diseases (babesiosis, borelliosis, tick fever) that cause encephalitis/meningoencephalitis, must be established from the clinical and paraclinical exams (blood specific tests).

A proper treatment started in due time is important because by not doing so the lesions of the nervous system may lead to the death of the patient.

The complications that can develop, especially in the case of babesiosis due to the adhesion of destroyed red blood cells or dead parasites to the vascular walls must be carefully monitored.

The owner must be informed that if complications develop the time needed for full recovery may be up to 6 weeks (if the treatment is followed accurately).

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# CASE STUDY – CANINE HEARTWORM DISEASE

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**Key words:** dog, heartworm,

**SUMMARY**

This paper presents the cases of five dogs that arrived in our clinic with mild general symptoms: tiredness, lack of breath, apathy, picky eating, and rare coughs. After taking the case history, the clinical and complementary exams, the diagnosis given was that of canine heartworm disease and the appropriate treatment was started.
During the spring and summer of this year (1.03 – 1.07.2010) in our practice there have been 5 different cases of canine heartworm disease. Given the case history and the clinical exams the dogs were sent for a hematology exam. Because of the signs and the laboratory results a blood sample exam that was taken between 6 p.m. and 8 p.m. (through direct exam, MGG colored smear or exam of the centrifugal sample) because we suspected the presence of the parasite.

## 1. MATERIALS AND METHODS

The cases were studied and treated at the Medical Clinic of the FMV, Bucharest; the clinical and neurological exams done in the Medical Clinics.

For these cases, the steps in diagnosis and treatment were as following:

***1.*** Case history
***2.*** Clinical exam
***3.*** Blood exam and biochemistry exam
***4.*** Serum exam (Dirofilaria)
***5.*** Radiologic exam

## 2. RESULTS AND DISCUSSIONS

The patients were 4 males (Golden retriever, German Sheppard, Caucasian Sheppard and one mix-breed) and one female (Romanian Sheppard).

The patients came into our practice for a check-up because the owners had noticed a slight change in their normal behavior (tiredness, lack of breath, picky eating, and cough from time to time).

All of the dogs were sent for hematology (slight anemia), biochemistry, parasitic, cardiac and ultrasonography exams.

Due to the lack of specific clinical signs, and the environment the patients live in (close to lakes and other bodies of water) we decided to test for dirofilariosis. This meant we took blood sample between 6 p.m. and 8 p.m. The result was positive for all five patients. Based on the clinical exams and the results of the laboratory tests all of the cases were classified as being "moderate".

This is a parasitic disease caused by infestation with *Dirofilaria immitis.* The infestation is spread by mosquito bite and the occasional case detected in newborns shows that this parasite can traverse the placenta.

The symptoms have a polymorph aspect with mild signs or signs of:

Cardiac deficiency syndrome (either compensated or not);

Nervous syndrome which may or may not appear;

Skin syndrome which also may or may not appear

Fortunately all of the cases that we took into account for this study did not present any of the above signs because the owners came to the practice at the first signs that there was something wrong with their dogs.

In two of the cases we also did a radiological exam of the thorax (latero-lateral), but no noticeable signs could be detected.

All of the patients underwent a cardiological exam (eco-cardiography and EKG), and the results were good, no adult parasites could be identified in the heart.

After the diagnosis was established we decided the following:

For prevention of pulmonary thromboembolism the patients were given 15mg/kg/day of softened aspirin, after a meal, 2 days prior to the administration of Immiticide; also administered was a dose of 5mg/kg/day of Doxicicline, for 10 days, starting 2 days before the Immiticide.

Immiticide (MELARSOMINE DIHYDROCHLORIDE) is an adulticide drug used as an antidote in doses of 2,5 mg/kg i.m. (or one 50mg phial for a 20kg dog) . The administration is repeated after 48 hours.

It is very important that this drug be injected deep into the paralumber muscles, at most 2ml for every place.

For suppression of the side effects of Immiticide it is advisable to use Dexametasone 0,25mg/kg i.m. or s.c. 30 minutes before the Immiticide.

The test for parasite infestation is repeated after 2 and 6 months from the last antidote dose.

If the follow-up test is negative after 2 months it is advisable to use Heartgard orally (Ivermectin/pyrantel) once a month from April until October, every year to prevent re-infestation.

The tests were negative after 2 months for every patient that we studied.

## 3. CONCLUSIONS

1. Given the fast evolution and progressive deterioration of the pathological process we can say that the accuracy and urgency of diagnosis determine the efficiency of treatment that must be started immediately.
2. The therapeutic approach to this disease must be started immediately after diagnosis, meaning as soon as the first clinical signs can be observed.
3. The clinical development of the pathological process postulates adjustments in the dynamics of treatment procedures, according to the degree of infestation and various complications of the disease.
4. The accuracy of specific anti-parasitic drugs limits the development of the pathological process and thus the patient's ability to recuperate (within the limits of comfort)
5. Regarding the specific drugs the utmost important thing is respecting the administration protocols (mainly the way and election spot – IMMITICE – is administered i.m., paravertebral)
6. For prophylactic reasons animal owners must be made aware regarding possible risks of infestation through mosquido bites when the dog has not been protected with profilactic measures

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# STUDY CONCERNING QUALITY CHARACTERISTICS OF TABLE EGGS FROM DIFFERENT HOUSING SYSTEMS

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**Keywords**: table eggs quality; quality characteristics; shell and content characteristics; housing system

## SUMMARY

In order to study the differences among commercial eggs from four housing systems i.e. cage, barn, free range and organic, a series of physical and parameters were evaluated on 40 fresh egg samples from the market. In the first series of analysis parameters, the highest air cell height and percentage of blood spots was measured for type 3 eggs (cage). Type 2 presented the highest percentage of meat spots, while type 0 had the highest values for albumen height, Haugh Unit and the highest value for yolk color. The pH of the samples was within ranges. In the second series of analysis parameters, the highest value of the egg weight was registered by type 1, comprising free-range eggs, while the lowest value was observed in type 0. The highest value for egg diameter and egg height were as it follows: type 2 with the largest egg diameter, and type 1 with the highest value for egg height. The percentage of cracked egg was highest in type 0. Egg shape index presented the highest value in type 2 eggs and the same type eggs had the highest percentage of albumen and yolk, while type 3 the highest percentage for shell. The thickest shell was observed in type 2 eggs (barn eggs), and the shell index value characterized organic eggs (type 0).

The European Union legislation is very strict about the importance of the housing system and the egg production. The introduction of the European Council Directive 1999/74/CE (EU, 1999a) set the minimum standards for the welfare protection of laying hens in cage, barn and free range housing systems. Regulation 1804/1999/CE (EU, 1999b) provides information about organic production methods for animal origin products and Regulation 2295/2003 (EU, 2003) suggests that the housing system must be designated on the box and on the egg shell. The codes to be used are 0 for organic production, 1 for free range, 2 for barn, and 3 for cage systems. Considering the cage system, starting from 2012, only eggs from hens housed in the so-called enriched cages (EU, 1999a), that is, those with at least 750 $cm^2$ of available space for hen, nest, litter and perches, will be allowed. Nevertheless the enriched cage system is not, at present, commonly implemented commercially. During the transition period, instead, the production of eggs from alternative systems (free range, barn, and organic) has been implemented. A consequence for this situation is that consumers face a very rich market

concerning the variety of eggs and egg products, but with different prices and in a lack of real information about the specific qualities of alternative eggs and cage eggs. Until now, a series of studies concerning the effect of the housing system on the characteristics of eggs were performed, especially on shell egg characteristics (Hauser & Folsch, 2002; Leyendecker et al., 2001a, 2001b; Sauveur, 1991; Van Den Brand, Parmentier & Kemp, 2004).

The aim of this study was to evaluate several characteristics of table eggs which might affect the final quality of this product, as influenced by the different housing system(cage, free range, barn, and organic), classified according to the EU legislation.

## 1. MATERIALS AND METHODS

Ten samples of commercial graded eggs for each type of housing system (0,1,2 and 3) were purchased from a local supermarket, at a period of 2-3 days from production date. The physical analyses were performed on yolk and whole egg. The shelling and the separation of the yolk from the albumen were made manually. The yolk was separated from the albumen and the vitelline membrane was removed using a spatula. All the eggs were individually weighed. Afterwards, the percentage of eggs bearing blood or meat spots was calculated by visual inspection. Air cell height (mm) was determined by measurement with a specific card. Albumen height was measured and the Haugh Units were estimated using Haugh equation. Yolk color was evaluated using a Roche fan. On the total egg content, pH was also measured using pH tape.

A second series of analyses targeted the following: egg weight (g), egg diameter (cm), egg height (cm) – using a manual calliper – , the percentage of cracked eggs by candling, egg shape index, the percentage of albumen, yolk and shell, shell thickness and shell index.

## 2. RESULTS AND DISCUSSION

The results were variable concerning the parameters for each type of table egg. The results are showed in tables 1 and 2, while the graphics for these values are included in figures 1, 2 and 3. Air cell height had the highest value registered for type 3 (cage eggs), while the lowest was observed at type 2, (barn eggs). The highest percentage of blood spots was observed at type 3 (cage eggs) and the highest for meat spots was

registered for type 2 (barn eggs). The pH values were normal for this type of product, with 7,51 up to 7,61 for cage eggs.

***Table 1***

**The first series of parameters selected for table eggs analysis**

| ***Evaluated parameter*** | ***Classification according to the housing system*** | | | |
|---|---|---|---|---|
| | ***0*** | ***1*** | ***2*** | ***3*** |
| **Air cell height (mm)** | 3,54 | 3,67 | 3,38 | 3,84 |
| **Blood spots (%)** | 9,24 | 3,35 | 8,47 | 11,29 |
| **Meat spots (%)** | 11,97 | 15,85 | 17,92 | 10,87 |
| **pH whole egg** | 7,57 | 7,51 | 7,52 | 7,61 |
| **Albumen height (mm)** | 5,33 | 5,29 | 5,15 | 4,79 |
| **Haugh Unit** | 68,8 | 67,2 | 68,1 | 62,3 |
| **Yolk color (Roche scale)** | 10,5 | 10,1 | 9,8 | 9,5 |

The albumen height, measured in millimeters, had the highest value in type 0 (organic) eggs, while the lowest one was observed in type 3, cage eggs. The Haugh Unit was 68,8 for type 0 (organic eggs), 67,2 for type 1 (free-range eggs), 68,1 for type 2 (barn eggs) and 62,3 for type 3 (cage eggs). The highest value concerning yolk color was obviously reserved for organic eggs, due to nutritional influence, while the lowest was observed in type 3 (cage eggs).

***Table 2***

**The second series of parameters selected for table eggs analysis**

| ***Evaluated parameter*** | ***Classification according to the housing system*** | | | |
|---|---|---|---|---|
| | ***0*** | ***1*** | ***2*** | ***3*** |
| **Egg weight (g)** | 63,8 | 67,1 | 64,2 | 65,4 |
| **Egg diameter (cm)** | 4,21 | 4,48 | 4,52 | 4,44 |
| **Egg height (cm)** | 5,78 | 6,54 | 5,91 | 5,74 |
| **Cracked eggs (%)** | 15 | 11 | 12 | 7 |
| **Egg shape index (%)** | 75,7 | 74,8 | 76,9 | 75,5 |
| **Albumen (%)** | 65,8 | 65,2 | 65,7 | 64,3 |
| **Yolk (%)** | 23,7 | 24,6 | 25,3 | 24,8 |
| **Shell (%)** | 10,5 | 10,2 | 9 | 10,9 |
| **Shell thickness (mm)** | 0,45 | 0,51 | 0,52 | 0,47 |
| **Shell index (g/cm$^2$)** | 0,284 | 0,267 | 0,264 | 0,252 |

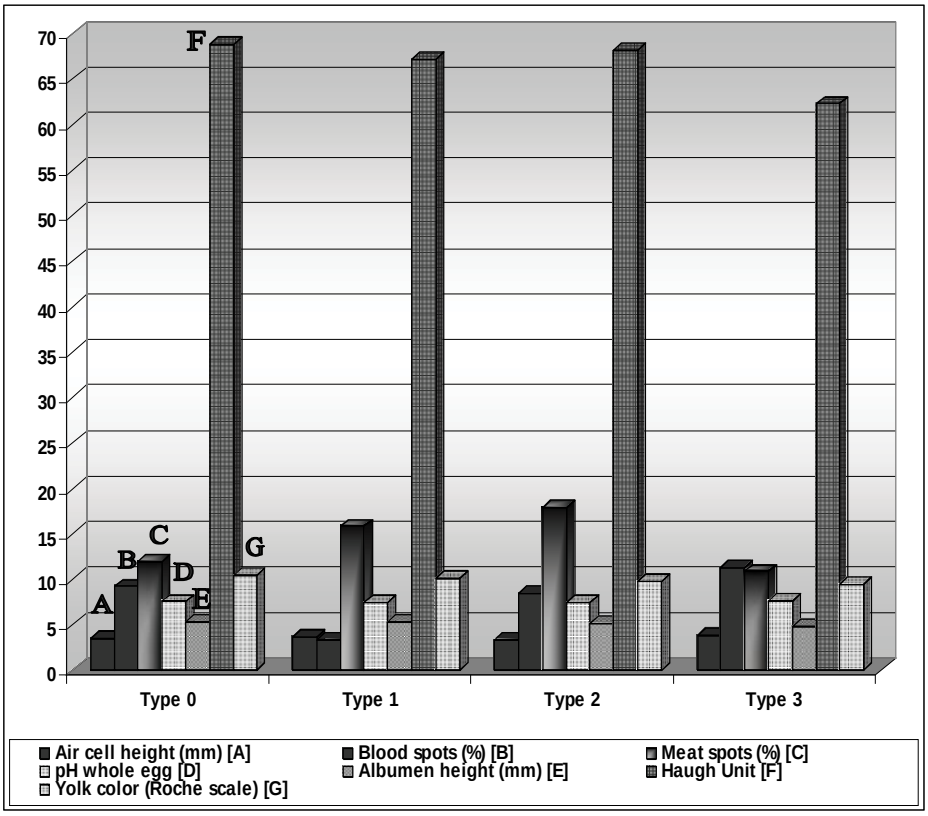


**Fig. 1 – The mean values for the first series of parameters selected for analysis of table eggs from different types of housing systems**

Concerning the egg weight, the highest value was registered by type 1, comprising free-range eggs, while the lowest value was observed in type 0. The highest value for egg diameter and egg height were as it follows: type 2 with the largest egg diameter, and type 1 with the highest value for egg height. The percentage of cracked egg was lowest in type 3 and highest in type 0. Egg shape index presented the highest value in type 2 eggs, while the lowest was observed in type 3.

Type 2 eggs had the highest percentage of albumen and yolk, while type 3 the highest percentage for shell. The thickest shell was observed in type 2 eggs (barn eggs), and the shell index value characterized organic eggs (type 0).

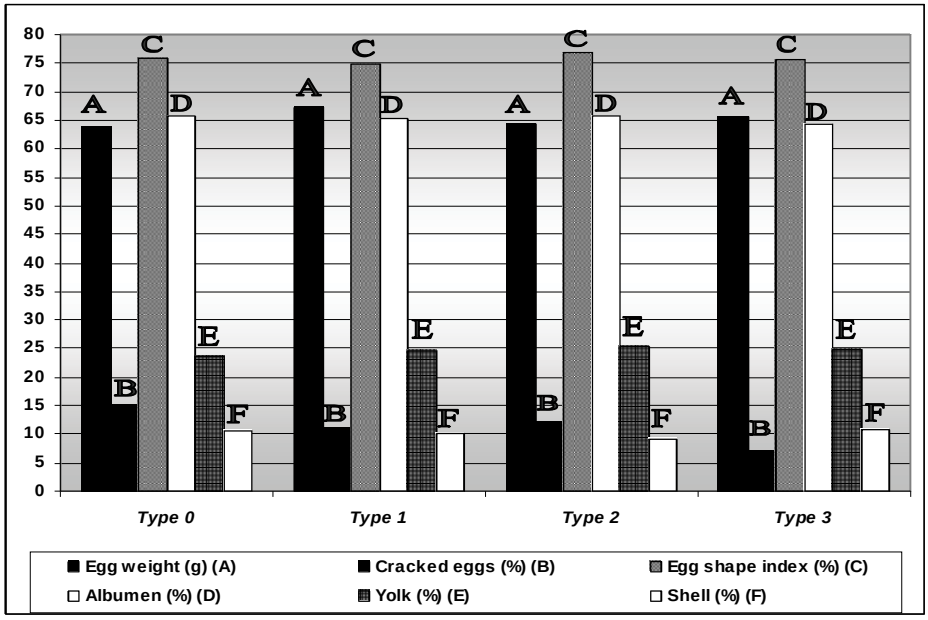


**Fig. 2 – The mean values for the second series of parameters selected for egg analysis (1)**

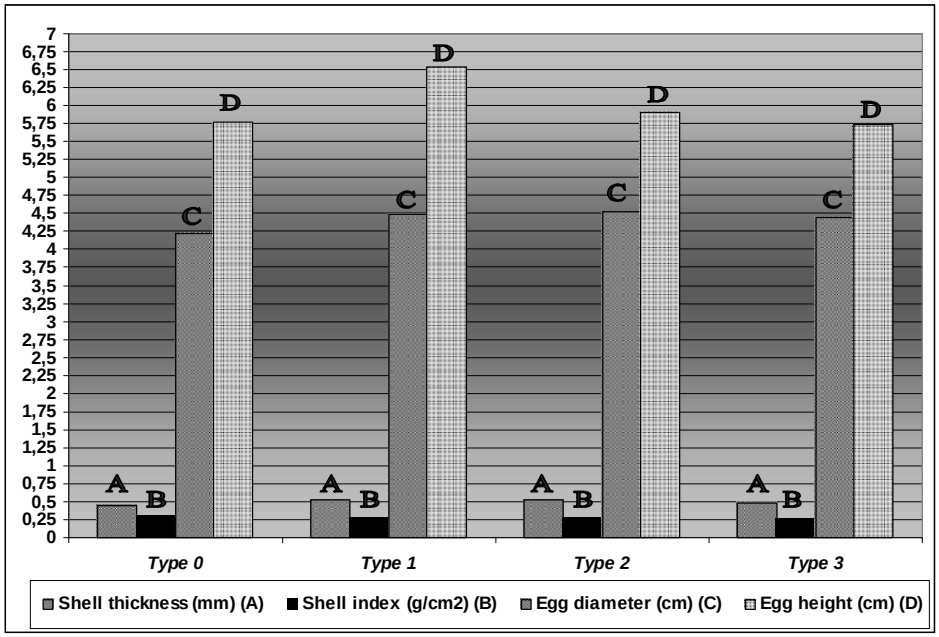


**Fig. 3 – The mean values for the second series of parameters selected for egg analysis (2)**

## 3. CONCLUSIONS

The analysis of several characteristics of table eggs put on the market as classified concerning the EU legislation led to results that show normal values. This is proof that commercial table eggs are within ranges concerning quality and that consumers may buy and use them as a safe nutritional resource.

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# HEPATOPATHIES IN BROILER CHICKENS: MORPHOLOGY, ETIOLOGY AND IMPLICATIONS IN FOOD SAFETY

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**Key words:** hepatopathies, broiler, food safety

**SUMMARY**

The hepatic lesions in broiler chickens are considered to be an important source of economic loss in the poultry industry.
The increased demands of the consumer regarding the food safety and quality impose the poultry processors to monitories the entire technological flow by applying the "from farm to table" principle.
The aim of this investigation was to link different types of hepatic lesions from young slaughtered poultry liver with the presence of pathogenic and conditional pathogenic microorganisms.
411 livers were, grossly, examined and two types of lesions were observed: non-specific hepatopathy with diffuse degenerative features associated with vascular reaction (94.4% of samples) and multifocal miliary necrotic hepatitis (5.6% of samples).
22 organs with representative gross lesions were submitted to cytological (May Grűnwald Giemsa stain), histological (Masson trichromic and Congo red stains) and microbiological exams.
Miliary necrotic hepatitis was associated with *Pseudomonas spp.* (5/7) while non-specific hepatopathies was correlated with *E. coli* (8/15), yeasts (8/15) and *Enterococcus spp*. (7/15).

The liver is the most important organ involved in metabolic processes of the body and it is considered to be one of the most eloquent witnesses of any disease, as it is the subject of different types of etiologic attacks: infectious, toxic, metabolic, nutritional, traumatic (Doneley B., 2004).

Sometimes, minor disturbances in liver function cause localized gross and microscopic changes in the liver, but with visibility throughout the body.

The typology of liver lesions, correlated with the isolation of microorganisms, can provide information about general health status of the flock and the potential damage to consumers' health.

As diagnostic methods have improved, veterinarians have become aware of the presence of liver disease in poultry for slaughter. Diagnosis of liver disease involves a combination of factors based on clinical

history, examination, diagnostic imaging and laboratory (Wade, L. 2008).

The increasing consumer's demands require the slaughterhouses' owners and processors of poultry meat to provide high quality and hygienically safe products. Thus careful monitoring should be imposed on all factors, the process flow, which could in any way affect the finished product, by applying the principle "from farm to table". (Hutu I. et al., 2004). In a study conducted in a medium-sized slaughterhouse in the Netherlands revealed the next consideration "*Condemnation of carcasses in … poultry with hepatic lesions has been mentioned to contribute to one-fifth of the economic loss in broiler industry*" (Lovland A et al., 2001). Also, a number of studies have been conducted to determine the connection between different diseases in broiler and the presence of microorganisms (Nakamura K. et al., 1999, Sasaki J et al., 2000, Supartika I. et al., 2007).

## MATERIAL AND METHODS

Harvested 411 livers, weighing 18.4 kg, from young slaughtered poultry have been grossly examinated.

For more specific exams, 22 organs were selected, considered to be representative as gross lesional expression.

There were performed cytological exam (May Grünwald Giemsa stain), histological exam (Masson trichromic and Red Congo stain) and microbiological exam (selective culture media - CIN (Schiemann) agar, Wauters agar, Mac Conkey agar, Christensen media, Simmons agar).

## RESULTS AND DISCUSSION

Based on the observed hepatical changes, two major types of gross lesions have been observed: nonspecific hepatopathy with diffuse degenerative character associated with different intensity of vascular reactions and multifocal miliary necrotic hepatitis (Fig. 1 and Fig. 2).

Nonspecific hepatic lesions (diffuse degenerative features associated or not with vascular disorders) were representative for 388 livers, 16.9kg (94.4% of livers rejected), while multifocal miliary necrotic hepatitis encountered in 23 livers, 1.5 kg (5.6% of livers rejected).

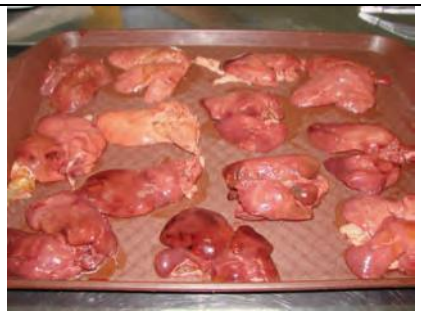

**Fig. 1 Nonspecific hepatopathy with diffuse degenerative character associated with different intensity of vascular reactions. Gross feature**

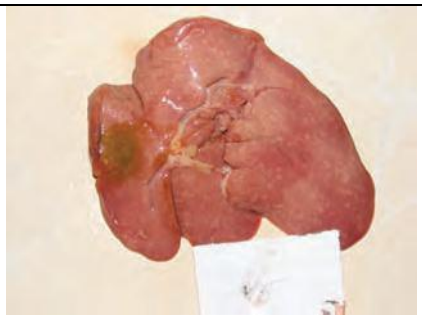

**Fig. 2 Multifocal miliary necrotic hepatitis. Gross feature**

Cytological, histopathological and microbiological exams were preformatted for 22 livers (15 livers for non-specific hepatopathy and 7 livers for multifocal miliary necrotic hepatitis).

Cytological, the degree of hepatocyte degeneration and the typology of inflammatory cells were examinated. Nonspecific hepatopathy was featured by erythrocytes, normal, degenerated or autolysis hepatocytes, many nude nuclei proving cell fragility. Multifocal miliary necrotic hepatitis expressed normal, degeneration or autolysis of hepatocytes, inconstant occurrence of heterophils and mononucleated cells being found.

Histologically, for nonspecific hepatopathies were dominantly featured by hepatocyte degeneration as hiperhydration and steatosis. (12/15) (Fig 3 and 4). Autolysis was recorded in 8/15 cases. Blood flow disturbances as congestion (6/15) and haemorrhages (4/15) were associated with necrotic foci in a single case. (Fig. 5 and 6). Colangitis (4/15) was associated with the occurrence of lymphoid nodules (1/15) and congestion.

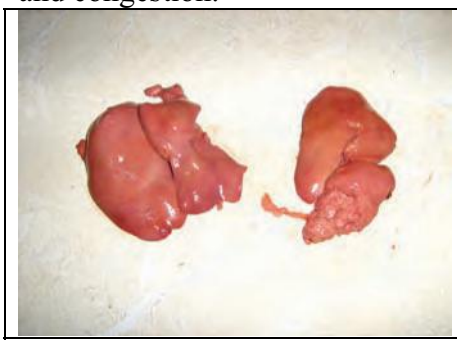

**Fig. 3 Hepatosteatosis- Gross feature**

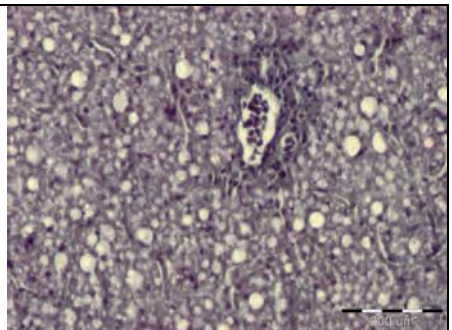

**Fig. 4 Hepatosteatosis – (Masson trichromic, ob. 40)**

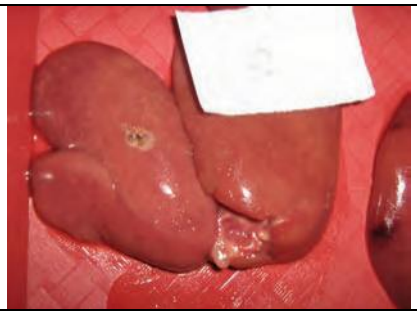

**Fig. 5 Hepatosis and necrotic foci. Gross feature**

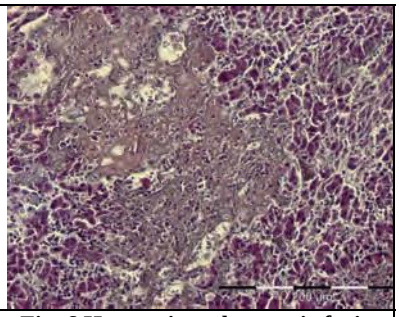

**Fig. 6 Hepatosis and necrotic foci, (Masson trichromic, ob. 20)**

Multifocal miliary necrotic hepatitis was histologically expressed as coagulation necrosis (6/7) and diffuse mononuclear infiltration (5/7). Granulomas were recorded in 2 cases. Amyloidosis (4/7) was correlated with necrotic foci, diffuse mononuclear infiltration and granulomas (Fig. 7 and 8).

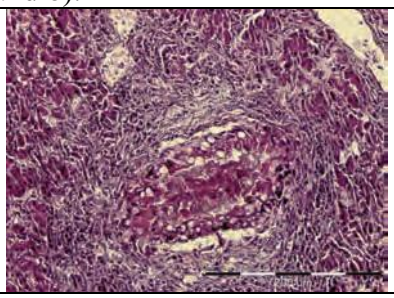

**Fig. 7 Granulomatous hepatitis (Masson trichromic, ob. 20)**

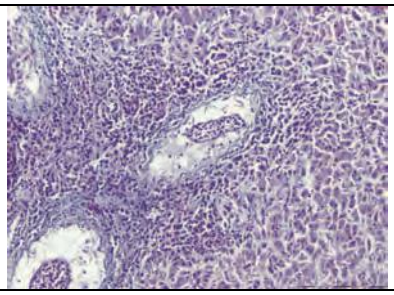

**Fig. 8 Diffuse mononuclear infiltration (Masson trichromic, ob. 20)**

Gross and microscopically features were correlated for nonspecific hepatopathies and multifocal miliary necrotic hepatitis. (Fig 9 and 10).

Total microbiological species from the depth of the liver tissue were evaluated. The most common microorganisms were *E. coli*, *Pseudomonas spp.* and yeasts.

*Pseudomonas spp.*, yeasts and coliphormes were isolated from nonspecific hepatopathies group, histologically diagnostic as colangitis (Fig. 11). Multifocal miliary necrotic hepatitis were microbiologically expressed as *Pseudomonas spp.* and yeasts isolates (Fig. 12).

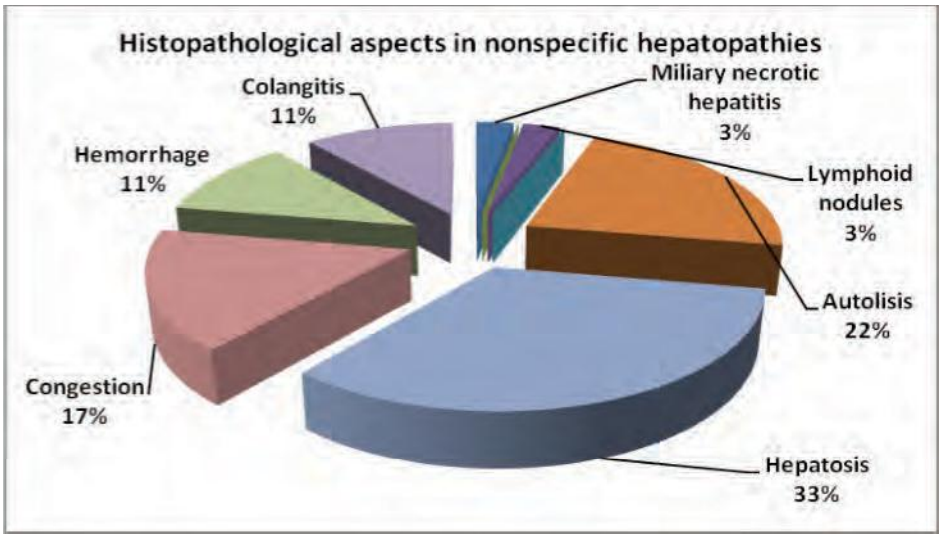


**Fig. 9 Correlation between gross and microscopic features – nonspecific hepatopathies**

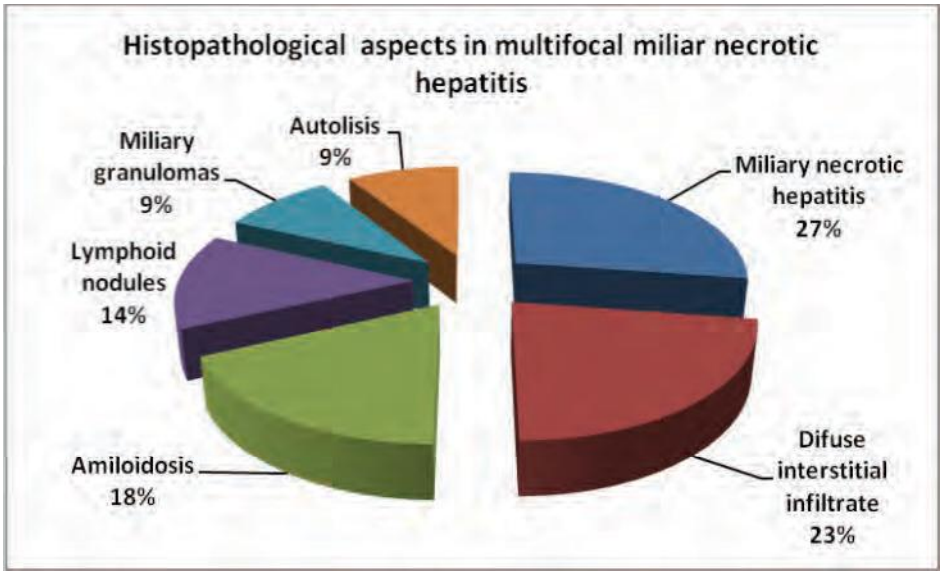


**Fig. 10 Correlation between gross and microscopic features – multifocal miliary necrotic hepatitis**

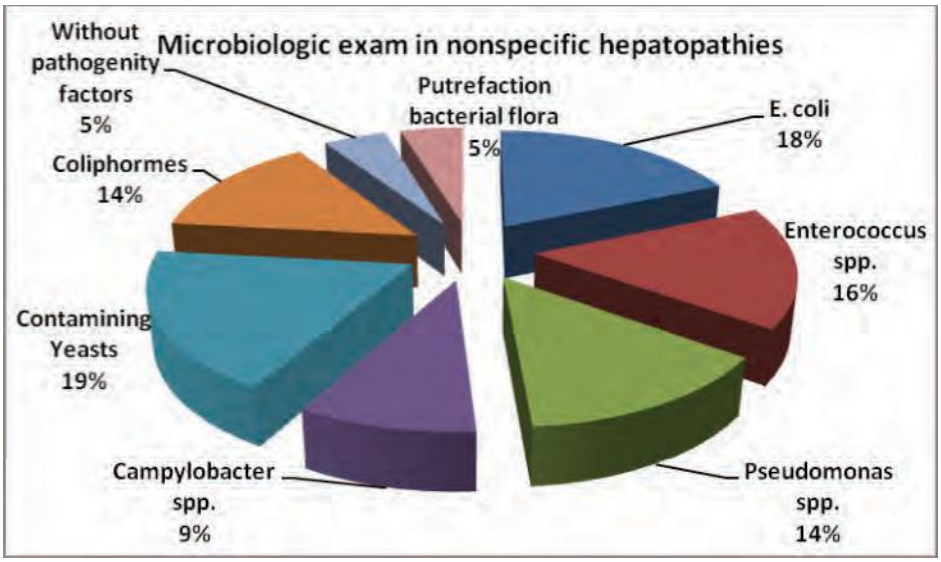


**Fig. 11 Correlation between microbiological exam and gross features – nonspecific hepatopathies**

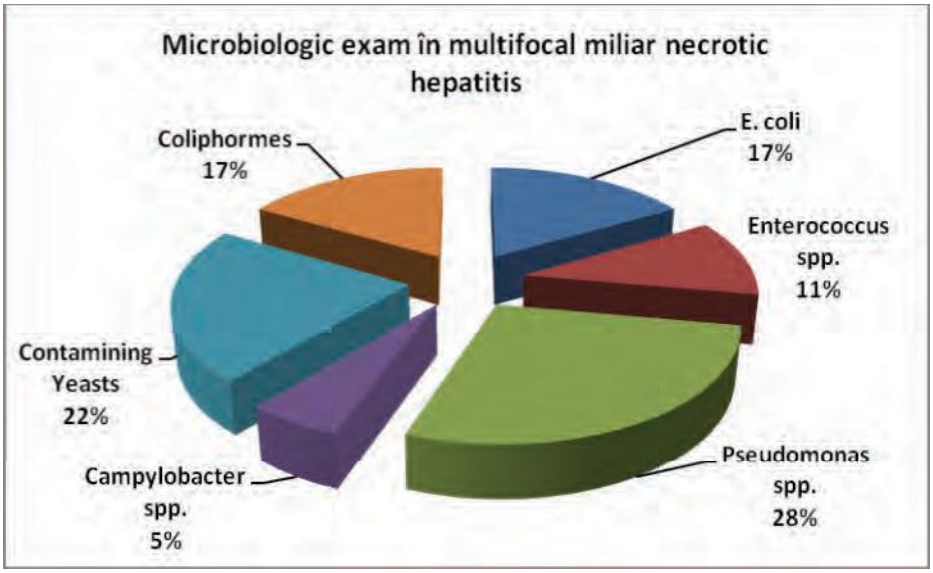


**Fig. 12 Correlation between microbiologic exam and gross features – multifocal miliary necrotic hepatitis**

Discussions are focused on the origin of the microorganisms isolated from the liver samples and their actions at hepatic level.

Several types of bacterial colonies (*Pseudomonas spp., E. coli, Enterococcus spp.)* were isolated from the same liver sample.

*E. coli* and *Enterococcus spp*. could belong to the normal bacterial flora of the gastrointestinal tract. (Supartika et al., 2007).

*Pseudomonas spp.* may come from the infested water. A very high level of this bacteria is commonly found in poultry houses and it has the ability to thrive in water systems. (Watkins et al., 2008).

All the bacteria can come from the wet litter used in the poultry farms. The presence of wet litter is the major cause of intestinal pathology which can be related with liver lesion (Hermans et al., 2006).

The mechanism of microbial translocation from the intestinal level to liver is relatively simple. Exposure to various stressors (e.g. nutritional, physical, toxic factors) can determinate an increased enteric epithelial permeability, which allow penetration of bacteria into mucosa and colonization of liver via blood flow.

Intestinal origin bacteria and their products are subsequently distributed to Kupffer cells and hepatocytes (Supartika et al, 2007). At this level inflammatory mediators are produced and activate heterophils.

The granulomas can be produced by E. coli, which can survive within macrophages without inducing cell death. E. coli can activate the production of macrophages and formation of granulomas.

Yeasts were isolated from the majority of the samples. Probably, these are contamination microorganisms coming from the slaughtering process, manipulation and transport of samples.

These results and correlations represent the first step in our study concerning hepatopathies in broiler chickens and food safety.

## CONCLUSIONS

3.1. 94.4% of samples were represented by non/specific hepatopathy and 5.6% by multifocal miliary necrotic hepatitis.

3.2. Hepatocyte degeneration as hiperhydration and steatosis were dominant for nonspecific hepatopathies (12/15); autolysis was recorded in 8/15 cases.

3.3. Coagulation necrosis (6/7) and diffuse mononuclear infiltration (5/7) were the dominant lesions for multifocal miliary necrotic hepatitis.

3.4. Miliary necrotic hepatitis is associated with *Pseudomonas spp.* (5/7)

3.5. Non-specific hepatopathies are correlated with *E. coli* (8/15), yeasts (8/15) and *Enterococcus spp.* (7/15).

## ACKNOWLEDGMENTS

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# MRI DIAGNOSTIC IN 10 DOGS WITH SEIZURE

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**Key words:** seizure, MRI, dog

**SUMMARY**

In order to diagnose some cerebral processes in dogs we use, beside clinical and radiological exam, the most modern imagistic technique, the MRI. This paper is the first study made in Bucharest and describes clinical and imagistic aspects in 10 dogs with clinical appearances of epileptically crises. 3 of the dogs showed no imagistic cerebral modifications and at the other 7 had the next pathological findings: 2 dogs with encephalitis, 2 dogs with glioma, one with ventricular asymmetry, one with pituitary adenoma and one with meningioma.

The most common brain pathology in dogs over 5 years with seizure includes neoplasia, vascular and degenerative disorders and inflammations (meningitis, encephalitis or meningoencehalitis). Canine brain tumors are common in dogs and include primary neoplasia of the central nervous system and various metastatic cancers. Some of the most common tumors of the central nervous system originating from brain tissue are: meningiomas, astrocytomas, glioblastomas, oligodendriomas, choroid plexus papillomas, and pituitary adenomas. Two of the most important signs a dog may have an intracranial brain tumor are seizure and unexplained behavioral changes. MRI is recommended for diagnostic of intracranial disease in dogs with seizure, due to excellent soft tissue detail

## 1. MATERIALS AND METHODS

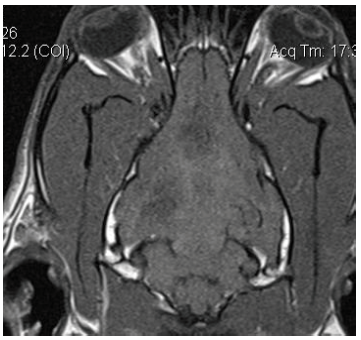
During January to June 2010 there were investigated thru radiological and MRI exam a number of 10 dogs, 8 males and 2 females, aged between 4 and 14 years old. Clinically, there have been observed seizures and one dog had visual deficiency (see case number 7). The radiological exam has been performed at the Röntgendiagnostic Service from the Faculty of Veterinary Medicine Bucharest and the MRI exam has been performed at Phoenix Diagnosis Clinic. Radiological exam was made in two incidences on the head area, ventro-dorsal (VD) and latero-lateral (LL), using a Philips Optimus machine with digital intake. For the MRI it was used a 1.5 Tesla machine (Avanto Tim-Erlangen, Germany) and the dogs were anesthetized first thru the classic method

(Acepromasine and Ketamine). All dogs were in sterno-abdominal recumbence. There were obtained weighted T1, T2 and T1 post-contrast (gadolinium) images in three perpendicular planes (sagital, axial and coronal).

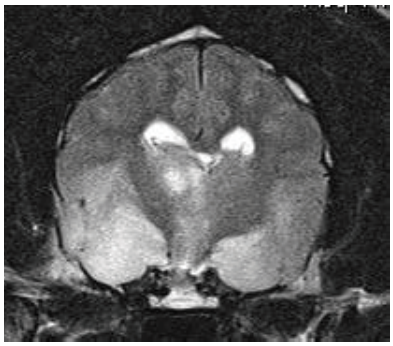
## 2. RESULTS AND DISCUSSIONS

The MRI revealed and there were diagnosed 3 types of cerebral pathologies: 4 dogs with intracranial tumors, 2 dogs with encephalitis, 1 with ventricular asymmetry. In the other 3 dogs examined MRI didn't revealed any modifications. Imaging characterization of the neoplasic processes was made after a strict correlation with bibliography without a histopathology for confirmation.

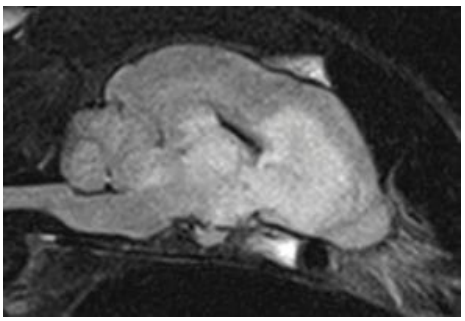
Case 1 - Boxer, 8 years, male. MRI reveals a diffuse lesion on the frontal right side, hyper signal in T2, hypo signal in T1, that doesn't fill with contrast. The lesion affects the cerebral cortex and the white substance.



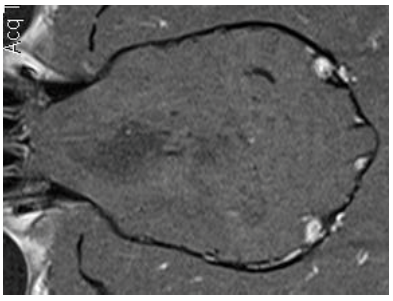
T1coronal



T2 axial



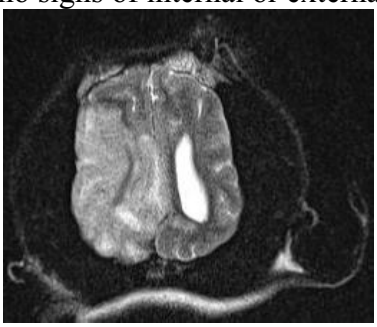
FLAIR



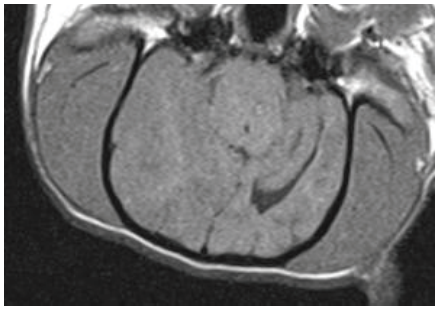
T1 contrast coronal

MRI reveals that a differential diagnostic must be made between a multifocal encephalic process and a tumor (multiple glioma).

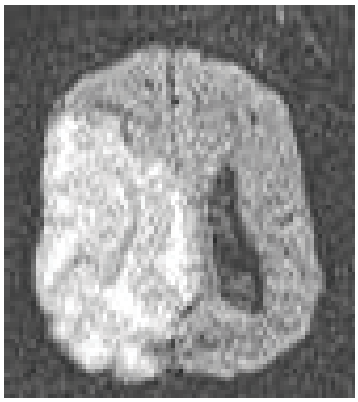
Case 2 - Common breed, 12 years, male. Cerebral MRI reveals important modifications with edematous aspect on the right cortico-subcortical and temporo-parieto-occipital side, enlargement of the cerebral winding and wiping intergyri spaces from this region. Right thalamus is also modified. Right lateral ventricle's temporal corn is compressed by the gyri edema and therefore thickened. Lateral ventricles don't have a mass effect, they keep their median place. A moderate mass effect is shown though on the cerebral brain stem. There are no signs of internal or external hydrocephaly.



T2 coronal



T1 axial



T1 contrast coronal

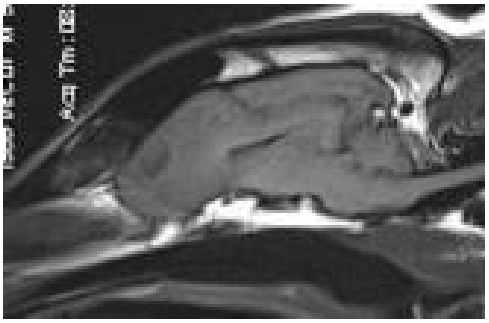
Intense modifications with right cortico-subcortical temporo-parieto-occipital and right thalamic edematous aspect (a differential diagnostic must be made between an encephalic processes a diffuse glioma).

Gliomas and meningiomas are the most common intracranial tumors seen in dogs. Glioma is a tumor with a development point in glial cells from the brain, spinal cord or optical nerve. In humans cerebral location is the most common. Considering the type of the glial cell that is affected gliomas are subdivided in ependiomas, astrocytomas and oligodendrogliomas. Astrocitomas are the most common neuroectodermic tumors; they represent 10% of the diagnosed primary tumors in dogs, brahicephalic breeds being the most affected. WHO

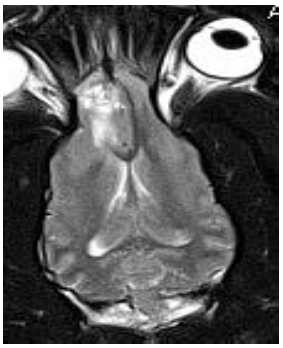
(World Health Organization) classified astrocitiomas after pathological evaluation in 4 classes, the last of them being glioblastoma, the most common in dogs an humans (5% from all astrocitomas) [7]. An MRI typically shows a brain stem glioma as an expansive, infiltrative tumor. They show as iso dense to slightly hypo dense on T1-weighted images and hyper dense on T2-weighted images. Use gadolinium enhanced T1-weighted, they are usually iso dense or hypo dense with ring enhancement. Edema, invasive character and slight definition of the margins define an aggressive character of malignity [5].

Case 3 - German shepherd, 12 years old, male. Tumoral mass, 13-15 mm with regular contour, relatively well defined in the right frontal eyeball in relation with falx cerebri. The mass has a scratchy structure and loads moderate and scratchy with the contrast substance i.v., doesn't present an obviously mass effect but there is though a perilesional edema. Symmetrical ventricular system with normal dimensions located on the median line. Starting point appears to be the meninges. Cerebrum and the brain stem appear to be without any pathological modifications.

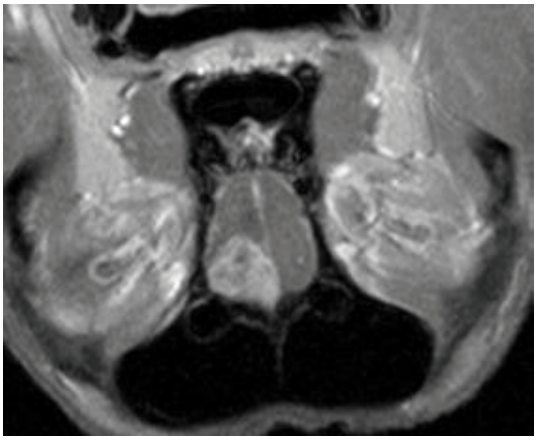
According to near meninges supratentorial location, well individualized, moderate hyper signal we could say it is a meningioma.



T1 sagital



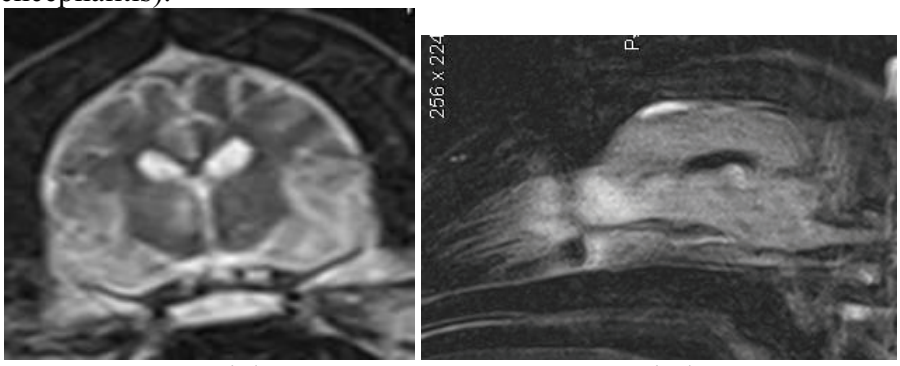
T2 coronal



T1 contrast axial

Meningiomas (arising from the meninges, a neural crest-derived cell) are the most common brain tumor of dogs, accounting for 35-45% of all primary brain tumors [5]. Golden Retrievers as well as other dolichocephalic (long-nosed) breeds are particularly susceptible.

Case 4 - Golden retriever 12 years, male. Cerebral MRI shows diffuse modifications of the signal, hyper signal T2 and FLAIR at the temporal lobes bilateral with extension to the frontal lobes. A lesion about 10mm with hyper signal T2 and FLAIR is also sown in the right side of the basal ganglia. There are no images of constituent abscesses. The ventricular system has normal dimensions, symmetrical located on the median line but there is bilateral sinusitis with liquid. MRI aspect of the lesion suggests most likely an inflammatory-infectious process (encephalitis).

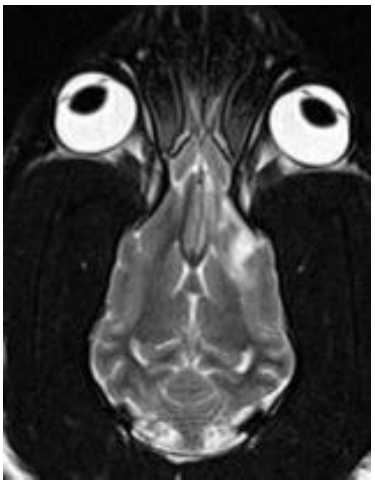



T2 axial FLAIR sagital

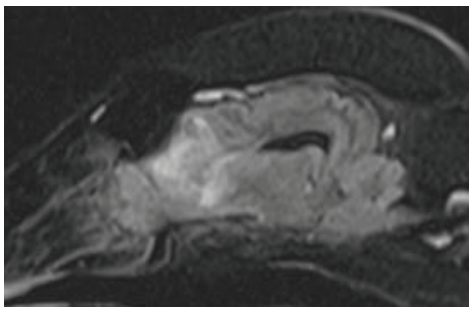
Case 5 - Pekinese 11 years and 7 months, male. MRI exam reveals diffuse signal modifications on the frontal on the left, hyper signal T2 and FLAIR, hypo signal T1 that involves the cerebral cortex too. At this level, in the area describe earlier we can see a contrast substance capture

on about 1 cm. There are no hemorrhagic lesions. No mass effect above adjacent structures. Another area with diffuse signal modifications can be seen in right cerebrum hemisphere next to be the acoustic-vestibular and facial nerve emerges on the right side. Modifications from this level don't load themselves with contrast substance. There are no areas of acute ischemia and no hemorrhagic intra or extra cerebral collections.

MRI aspect of the lesions (hyper signal T2 and FLAIR, hypo signal T1, moderate contrast substance enhancement) [9, 10] rise the problem of differential diagnosis between focal encephalitis, most likely, and secondary determinations, more or less. The symptoms were absent after the treatment so it confirms the encephalitis.

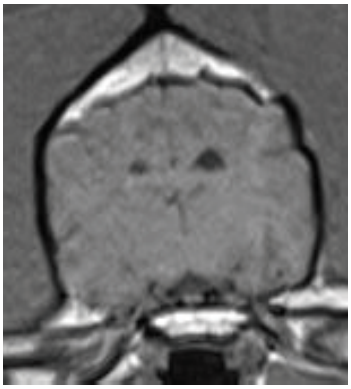


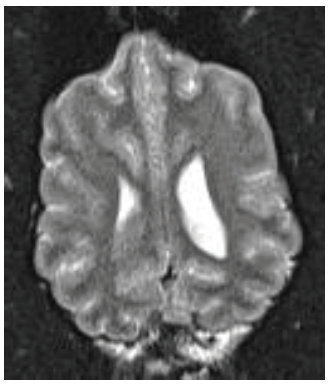
T2 coronal



FLAIR sagital

Case 6 - Common breed, 4 years, male. Right lateral ventricular system's asymmetry is with smaller dimensions than the left one. There are no signs of left lateral ventricle enlargement. There is no signal modification or images of tumors on the cerebral substance. There are no lesions of the brain stem. MRI cerebral aspect is normal.



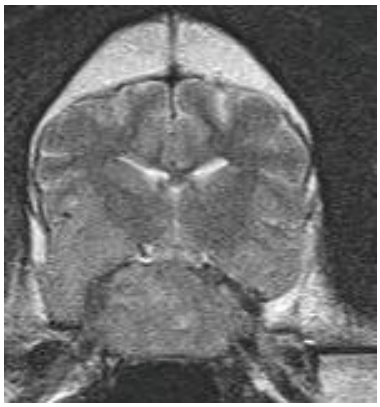


T1 axial T2 coronal

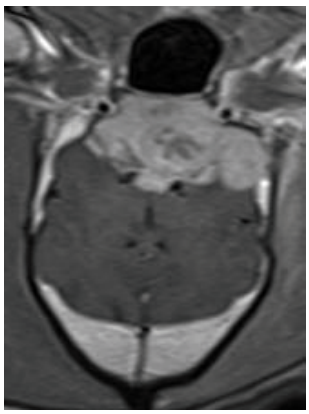
Slight asymmetry of the ventricular system (lateral right ventricle with smaller dimensions).

The asymmetry may be indicative of asymmetry of the temporal lobes. The asymmetry can also be the result of a prior insult (e.g., trauma, stroke, etc) to the brain. Other conditions that can cause the temporal horns to be asymmetric are hydrocephalus (e.g., increased cerebrospinal fluid in the ventricles) or increased edema (e.g., swelling of the brain). The Boxer had the relatively largest lateral ventricles, whereas in the other breeds, their sizes were very similar. Ki-Ja Lee and co-workers showed that 32 of the dogs investigated (64%) had symmetric lateral ventricles, and in 18 dogs (36%) they were asymmetric. Of these 18 cases, the left lateral ventricle was larger in 12 dogs (67%) [6]. Another study showed that eleven dogs had lateral ventricles classified as normal sized (0-14% Vh/Bh) while 10 of 21 dogs had moderate enlargement (15-25% Vh/Bh) of one or both lateral ventricles [4].

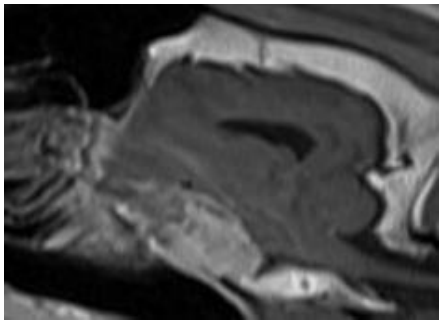
Case 7 - Boxer 11 years and 8 months. MRI reveals a large tumoral mass located at the base of the brain with the following dimensions 5 cm AP/2 cm CC /4 cm LL iso intense in T2 and FLAIR and loads itself with the contrast substance. It has a pituitary starting point most likely. The mass invades sphenoid bone to the pharyngeal mucosa and invades it. The mass comes in superior contact with the cerebral parenchyma, doesn't show signs of its invasion, and also comes in contact with carotid arteries and basilar trunk. The mass invades the cavernous sinuses and reaches the top of the orbits bilateral, most obviously on the left side with extension and invasion of the posterior extrinsic muscles of the eye balls. Diffuse tumoral infiltration on the left temporal muscle. There are no secondary intracerebral effects.



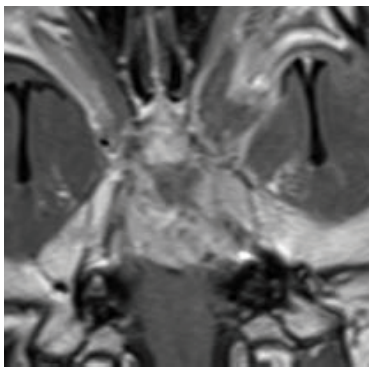
T2 axial



T1 contrast axial



T1 contrast sagital



T1 contrast coronal

Pituitary adenomas in humans are tumors that occur in the pituitary gland, and account for about 15% of intracranial neoplasms. They often remain undiagnosed, and small pituitary tumors have an estimated prevalence of 16.7% (14.4% in autopsy studies and 22.5% in radiologic studies) [2]. Visual difficulties arising from the compression of the optic nerve [1]. Pituitary gland tumors are very common in the canine. A productive form arising from the anterior pituitary is the primary cause of Cushing's disease of dogs [8].

## 3. CONCLUSIONS

3.1. Due to MRI multiplanar image acquisition capacity (sagital, coronal and axial) and a very good contrast it provides very close anatomical and morphological definitions of cerebral pathology.

3.2. The possibility of acquiring images in T1, T2, FLAIR and T1 post contrast may lead to a strict differential diagnose (inflammatory, circulatory and neoplazic processes, diffuse or well defined, located).

3.3. Due to its multitude of advantages and to its noninvasive, MRI represents the elected diagnosis and characterizing method of the tumor pathology.

3.4. Results are based on imagistic different characters of each pathology.

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# STUDY REGARDING THE IMPLEMENTATION OF THE *LEPTOSPIRA SPP.* ISOLATION AND CULTURING METHODS FROM PATHOLOGICAL MATERIAL FROM EXPERIMENTAL AND NATURALE INFECTED ANIMALS

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**Key words:** *Leptospira spp.* isolation and culturing, dark field microscopy

**SUMMARY**

The study consisted by the assimilation, implementation and optimisation of the methodology regarding the leptospires isolation and culturing, based on the recomandations of the OIE Terestrial Manual, edition 6, 2008.
Eight types of culture media was prepared, EMJH semi-solid with 4 combinations of antibiotics/chemotherapeutics which suppress contaminating bacteria, EMJH liquid, EMJH liquid with rabbit serum, Korthoff medium and Fletcher semi-solid medium.
The culturing was performed with pathological materials from exeprimental infected animals and the M1 type of the EMJH semi-solid, with 100µg/ml 5-fluorouracil, was the best culture media for the optimal growing of leptospires. The M3 type of the EMJH semi-solid, with 300 µg/ml 5-fluorouracil and 20 µg/ml nalidixic acid, inhibited strongly leptospiral growth and we obtained just 18,2% positive results from liver. For the M4 type of the EMJH semi-solid, with 100µg/ml 5- fluorouracil, 10 µg/ml rifampycin and 2µg/ml amphotericin B, we obtained 45,5% positive results from liver and 36,36% from kidneys, but the results were negative for all samples of fluid pericardic and peritoneal, probable caused by post mortem changes witch can rapidly reduce the number of viable leptospires.
We also perform the isolation and culturing of leptospires from urine and we obtained 18,2% positive results for M1 and M2, while for M3 and M4 the results were negative.
The dark field microscopy of the urine and kidneys cultures from a dog with positive serology, which was performed at 26 days after the inoculation of the culture media, was positive for the types M1, M2 and M4 of the EMJH semi-solid and negative for the type M3, which was negative even at 8 weeks.
We succed to isolate leptospires from patological materiales taken from an aborted animal foetus. The result was positive for both inoculations of the culture media, at 24 and 96 hours after the samples of the tissues were taken. So, if pathological materials are collected, stored and transported correct, leptospires can survive and the culturing and isolation of leptospires can be posible.

The isolation of leptospires from body fluids or internal organs (kidney, liver, lung, brain) of aborted fetuses is considered to be diagnostic of chronic leptospirosis of the mother and active infection of the fetus (6).

The succes of the isolation and culturing of leptospires depends on the pathological material and the stage of the disease. Also, the

culturing must be carry out before the antibiotic treatment. During the leptospiraemic phase, till about the day 10 after the onset of ilness, the most suitable materials for the culturing are blood, vascular organs (liver, kidneys and spleen) and cerebrospinal fluid. Kidney, liver, and brain are the most suitable post- mortem material in fatal cases of animal leptospirosis. The isolation of leptospires does also depend on the their density in these materials. The degree of succes achieved in isolating leptospires is also depend on the kind of media and how the culture tubes inoculates with tissue are manipulated. Culture tubes are usually prepared as 1:10. 1:100 and 1: 1000-fold dilutions (3).

If tissues or fluids cannot be transported promptly to the laboratory for leptospiral culture, the samples should be kept beetwen +2 + 5°C to prevent overgrowth with other bacteria and autolysis of tissue samples (6).

## 1. MATERIAL AND METHOD

MATERIALS:

Eight types of culture media was prepared

- Four types of EMJH semi-solid medium (Ellinghausen and McCullough, modified by Johnson and harris) (1):

**M1** - contain 100µg/ml 5-fluorouracil.

**M2** - contain 200µg/ml 5- fluorouracil.

**M3** - contain 300 µg/ml 5- fluorouracil şi 20 µg/ml nalidixic acid.

**M4** - contain 100µg/ml 5- fluorouracil, 10 µg/ml rifampycin, 2µg/ml amphotericin B.

- EMJH liquid medium (1).
- EMJH liquid medium with rabbit serum (3).
- Fletcher semi-solid medium (3).
- Korthoff liquid medium (5).
- Rabbits
- The panel of serovars of *Leptospira spp.* and reference hyperimmune antisera against serovars of *Leptospira spp*. (Royal Tropical Institute – International WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis): *pomona, tarassovi, icterohaemorrhagiae, canicola, hebdomadis, wolffi, hardjo, grippothyphosa, australis, autumnalis, ballum, bataviae, javanica, sejroe, saxcoebing, bulgarica.*

METHODS:

The *Leptospira spp*. isolation and culturing methods from pathological material on the EMJH, FLETCHER and KORTHOFF culture media

The culturing of leptospires are made by the inoculation of 0,6 ml blood and cerebrospinal fluid into several tubes, each containing 6 ml various media cultures (dilutin 1/10). Urine can be colected from live animals or post-mortem, in fatal cases of animals leptospirosis. Urine is added to a 30 ml bottle containing 10 ml of sterile Leptospira diluent. The amount of inoculum is 0,6 ml for each tube containing 6 ml of various media cultures. Culture tubes are usually prepared as 1:10, 1:100 and 1:1000-fold dilutions (3).

The culturing of leptospires are made from tissues by the aseptically collecting up to 0,5 cm from liver or 6 x 1 cm wedges of cortex and 1 x 1 cm wedges of medula from kidney. These tissue wedges are added to a sterile Stomacher bag containing 9 parts of leptospira diluent for 5 minute. The serial dilution of the supernatant ($10^{-2}$ and $10^{-3}$) are made in the same diluent.

The $10^{-2}$ and $10^{-3}$ dilutions are inoculated in 50 μl and 125 μl amounts into tubes with media cultures (2).

Also, serial dilutions as 1/10, 1/100 şi 1/1000, can be performed directly into tubes with medium (3).

Cultures are incubated at 29ºC to 30ºC and read by low power, dry, dark field microscopy every one or two weeks but these periods may be increased or decreased at the discretion of the specialist. Suspect tubes with other micro-organisms should be subculturing into fresh media. Suspect tubes with no discernible growth of contaminants are topped off with 0,5 ml of fresh medium, and the tube are gently agitated (4).

## 2. RESULTS AND DISCUSSIONS

*Leptospira spp*. isolation and culturing methods from pathological material from experimental infected animals

In order to implement and optimisate the *Leptospira spp*. isolation and culturing methods, four types of EMJH semi-solid culture media was prepared, with 4 combinations of antibiotics/chemotherapeutics which suppress contaminating bacteria. We also performed the experimental inoculation of rabitts with 11 serovars of *Leptospira spp.*

We estimated the base-line antibody titre against leptospires from rabbits by microscopic agglutination test (MAT) using 16 serovars of *Leptospira spp.*. The results were negative for all rabits. We also determinated the antigenic specificity for serovars which were inoculated on rabbits, by MAT using the reference hyperimmune antisera against homologous serovars of *Leptospira spp*.

The rabbits were phenotypicaly identificated and were inoculated with one serovar, according with the protocol of the Subcommittee on the Taxonomy of *Leptospira*.

We collected blood samples for detection of antibodies against leptospires and pathological material for isolation and culturing of leptospires on the culture media. We estimated antibody titres by the microscopic agglutination test using 16 serovars of the *Leptospira spp.* Results are shown in the table no. 1.

In order to perform the microbiological culture we collected samples from liver, kidneys, the pericardic and peritoneal fluid and urine.

These samples were inoculated on the four types of the EMJH semi-solid culture media (M1, M2, M3 AND M4).

The cultures were incubated at 29ºC - 30ºC and were periodicaly examinated by dark field microscopy. We visualisated many bacteria with morphology, size and motility like leptospires in the positive cultures.

Results are shown in the figure no. 1.

***Table no. 1.***

**The determination of the imune response of the experimental infected rabbits**

| Serovar used in MAT / Serovar inoculated | Pomona | Icterо | Canicola | Wolffi | Hardjo | Grippo | Australis | Autumnalis | Ballum | Bataviae | Tarassovi |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Serum from rabbits which were inoculated with serovars of *Leptospira spp.* | | | | | | | | | | |
| **Pomona** | **6400** | - | - | - | - | - | - | 400 | - | 200 | - |
| **Ictero** | 200 | **12800** | 400 | - | - | - | 200 | - | 200 | - | - |
| **Canicola** | - | 800 | **12800** | - | - | - | - | - | 800 | - | - |
| **Wolffi** | - | 200 | 400 | **25600** | 12800 | - | - | - | - | 800 | - |
| **Hardjo** | - | - | - | 12800 | **25600** | - | - | - | - | - | - |
| **Grippo** | 200 | 100 | - | - | - | **25600** | - | - | - | - | - |
| **Australis** | 1600 | 1600 | - | - | 800 | 200 | **102400** | 3200 | 400 | - | 1600 |
| **Autumnalis** | 100 | 200 | - | - | - | - | 800 | **25600** | - | - | - |
| **Ballum** | - | 3200 | 3200 | 100 | 100 | - | - | - | **51200** | 200 | 800 |
| **Bataviae** | - | 200 | 400 | 400 | - | - | 200 | - | 400 | **25600** | 200 |
| **Sejroe** | - | - | - | 3200 | 3200 | - | - | - | - | - | - |
| **Tarassovi** | - | - | - | - | - | - | - | - | - | - | **12800** |
| **Hebdomadis** | - | - | - | 800 | 1600 | - | - | 400 | 400 | - | - |
| **Javanica** | - | 800 | - | - | - | - | 1600 | 1600 | 1600 | 400 | 6400 |
| **Saxcoebing** | - | - | - | 1600 | 3200 | - | - | - | - | - | - |
| **Nicolaevo** | - | 400 | - | - | - | 200 | 400 | 6400 | - | - | |

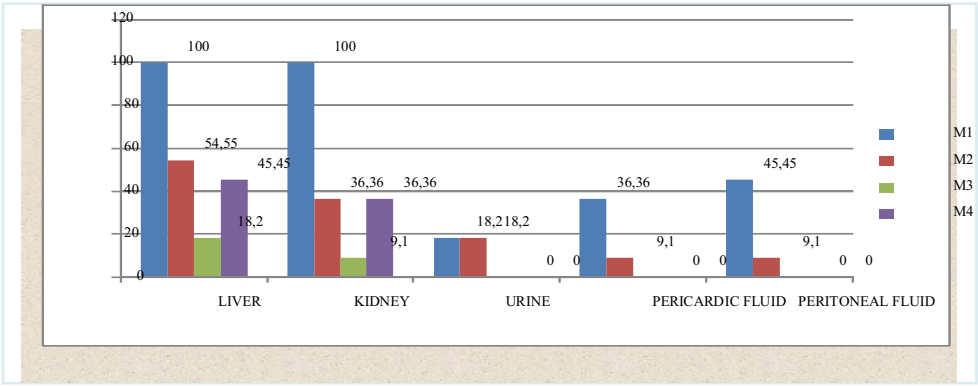


**Fig. no. 1. Results of the culturing leptospires on the EMJH semi-solid media (M1,M2,M3,M4)**

*Leptospira spp*. isolation and culturing methods from pathological material from naturale infected animals

We carred out the isolation and culturing of leptospires from a suspected dog of leptospirosis INFECTION. Leptospirosis was manifested by listlessness, drowsiness, jaundice, vomition and hematuria.

The serological exam performed by microscopic agglutination test was positive (1/100 – *L. icterohaemorrhagiae*; 1/50 – *L. canicola).*

After the dog died we collected the pathological materials, kidney and urine, in order to perform bacteriological exam.

We carred out the dark ground microscopic examination of the kidney and urine and we visualisated rarely bacteria with morphology, size and motility like leptospires.

We also performed the culturing of leptospires by the inoculation of the pathological materials on four types of the EMJH semi-solid culture medium (M1, M2, M3 and M4) preparing 1/10, 1/100 and 1/1000 fold-dilutions from these materials. The cultures were incubated at 29ºC - 30ºC and were periodicaly examinated.

A drop of cultures was placed on a clean glass microscope slide and examined for the presence of leptospires by the dark field microscopy.

We identificated bacteria with morphology, size and motility like leptospires, by the dark field microscopy, at 26 days from the inoculation of the material on the type M1, M2 and M4 of the EMJH semi-solid culture medium.

Subsequent, we performed successive subculturing in order to purificate and multiplicate of the cultures.

The exam was negative for the type M3 of the EMJH semi-solid culture medium, even at 8 weeks after the inoculation of the material into culture media.

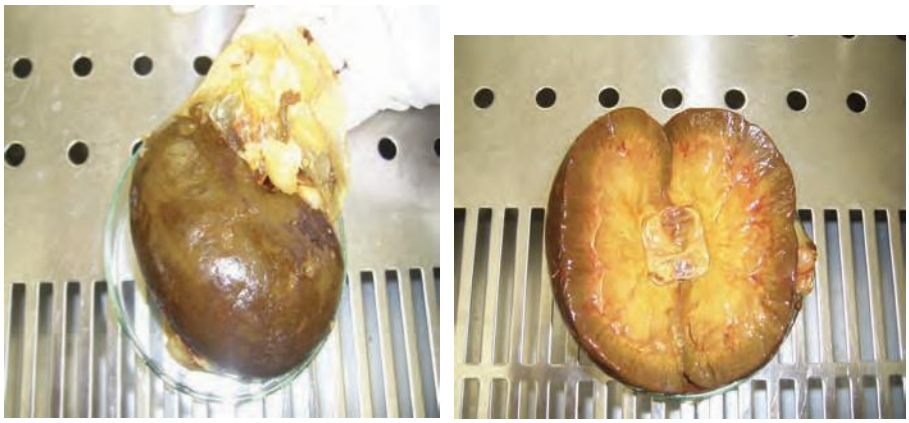

**Fig. no. 2. The post mortem exam of the kidney collected from an infected dog with serovars *L. icterohaemorrhagiae***

We also carred out the isolation and culturing of leptospires from an aborted cattle foetus.

These cattle came from a herd of pregnant cows with serological positive results by MAT.

Initial, the serological test performed at 14 days before abortion was negative. However, after abortion, the serological test was positive (*L. hardjo* – 1/1600; *L. wolffi* – 1/200, *L. australis* 1/200 şi *L. sejroe* – 1/100).

We collected pathological materials (brain, kidney, pericardic and peritoneal fluid) from aborted cattle foetus, witch were transpoted into sterile vessels of glass in order to performed the bacteriological exam.

The culturing was performe by the inoculation of the brain, kidney, pericardic and peritoneal fluid samples on several types of culture media, at 24 and 96 hours after abortion. Also, the culture tubs were prepared as 1/10, 1/100 and 1/1000 fold-dilutions for each sample.

The cultures were incubated at 29ºC - 30ºC.

The dark field microscopy was performed at 11 days after inoculation of the culture media by placing a drop of cultures on a clean glass microscope slide and we identificatied rare bacteria with morphology, size and motility like leptospires, aspects witch are shown in the figure number 4.

Subsequent, we performed successive subculturing in order to purificate and multiplicate of the cultures.

We carred out the dark field microscopy and identificated many bacteria with morphology, size and motility like leptospires, aspects witch are shown in the figure number 5.

The culturing exam results are shown in the table number 2 and the figure number 3.

***Table no. 2.***

**The culturing results - aborted cattle foetus**

| CULTURE MEDIUM | Diluţia materialului patologic | MATERIAL PATOLOGIC | | | |
|---|---|---|---|---|---|
| | | RINICHI | CREER | LICHID PERICARDIC | LICHID PERITONEAL |
| FLETCHER (at 24 hours after abortion) | 1/10 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/100 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| | 1/1000 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| FLETCHER (at 96 hours after abortion) | 1/10 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/100 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| | 1/1000 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| EMJH M1 (at 96 hours after abortion) | 1/10 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/100 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| | 1/1000 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| EMJH lichid (at 96 hours after abortion) | 1/10 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/100 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/1000 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| EMJH lichid ser (at 96 hours after abortion) | 1/10 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/100 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| | 1/1000 | POZITIVE | POZITIVE | NEGATIVE | NEGATIVE |
| KORTHOFF (at 96 hours after abortion) | 1/10 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/100 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| | 1/1000 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |

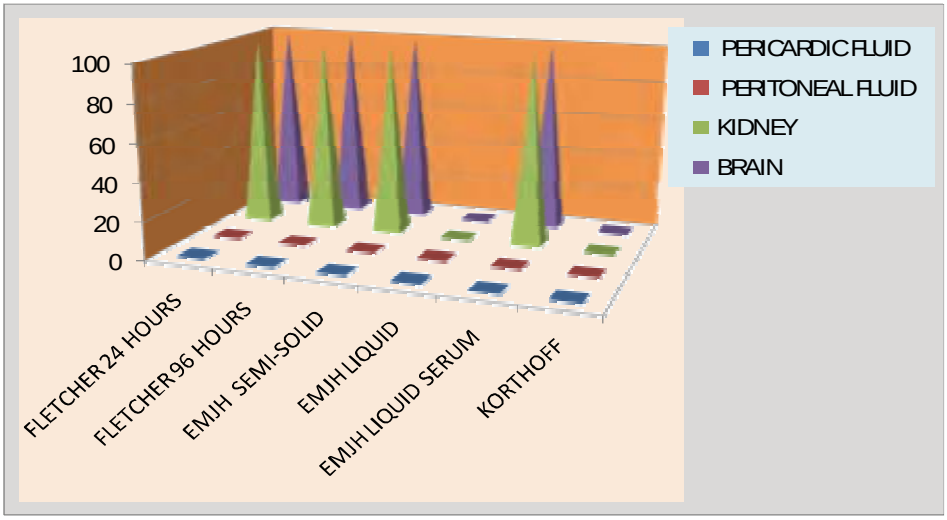


**Fig. no. 3. The culturing results performed of the pathological materials colected from an aborted cattle foetus**

**Fig. no. 4. The dark field microscopy performed from the primary cultures of leptospires**

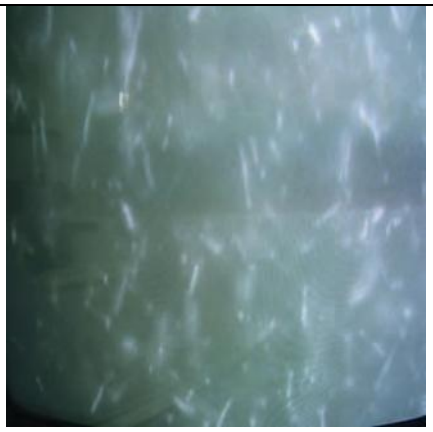

**Fig. no. 5. The dark field microscopy performed from the subcultures of leptospires**

## 3. CONCLUSIONS

3.1. The M1 type of the EMJH semi-solid, with 100µg/ml 5-fluorouracil, was the best culture medium for the optimal growing of leptospires with 100% positive results from liver and kidney. The M3 type of the EMJH semi-solid, with 300 µg/ml 5- fluorouracil and 20 µg/ml nalidixic acid, inhibited strongly leptospiral growth and we

obtained just 18,2% positive results from liver. For the M4 type of the EMJH semi-solid, with 100µg/ml 5- fluorouracil, 10 µg/ml rifampycin and 2µg/ml amphotericin B, we obtained 45,5% positive results from liver and 36,36% from kidney, but the results were negative for all samples of the pericardic and peritoneal fluid, probable caused by post mortem changes witch can rapidly reduce the number of viable leptospires. We also perform the isolation and culturing of leptospires from urine and we obtained 18,2% positive results for M1 and M2, while for M3 and M4 the results were negative.

3.2. The dark field microscopy of the urine and kidney cultures, which was performed at 26 days after the inoculation of the culture media with materials from a dog with positive results by MAT, was positive for the M1, M2 and M4 types of the EMJH semi-solid and negative for the M3 type . The exam was negative for the type M3 of the EMJH semi-solid culture medium, even at 8 weeks after the inoculation of the material into culture media.

3.3. The culturing of leptospires from an aborted cattle foetus (brain, kidney) was positive for both inoculums, which was performed at 24 and 96 houers after abortion. The results were positive for the M1 type of the EMJH semi-solid, with 100µg/ml 5-fluorouracil, EMJH liquid medium with rabbit serum and for the Fletcher medium and negative for EMJH liquid medium without rabbit serum and for the Korthoff medium.

Also, we noticed that, the culture tubs which were prepared as 1/10 dilution, were negative, probable because of the post mortem changes which may affect the viability and inhibit growth and multiplication of leptospires in culture media. The culturing of leptospires performed from the pericardic and peritoneal fluid was negative for both inoculums, which were performed at 24 and 96 hours after abortion.

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# INTRATUMORAL ADMINISTRATION OF GROSS FUNGI EXTRACT OF T3-2 STRAINS OF CLAVICEPS PURPUREA FOR ANTINEOPLASTIC EFFECT - CASE STUDY

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**Keywords** - Claviceps purpurea, fungus, antitumor

**SUMMARY**

Claviceps purpurea is a fitoparazite fungus from Clavicipitaceae family, genus Claviceps, whose parasitize different grains, especially rye, hence was called "ergot." In general ergots containing alkaloids. Some alkaloids are partial agonists, while others are antagonists, affecting both serotonin and catecholamines.
Representatives are clavinics alkaloids: agroclavie, festuclavie, elimoclavine. Ergot alkaloids *and their derivates* have either agonist or antagonist activity at different receptors: adrenergic, serotonin and dopamine. Ergot alkaloids and their derivates have the ability to inhibit the growth of certain hormone-dependent tumors by inhibiting the secretion of prolactine from the anterior pituitary gland.

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Representatives clavinic alkaloids are: agroclavina, elimoclavina and festuclavina.

Ergot alkaloids and their derivatives have the ability to inhibit the growth of certain hormone-dependent tumors by inhibiting the secretion of prolactin from the anterior pituitary gland (Cassady and Floss, 1977).

Treatment of prolactinoma, a thymus gland adenoma, and other tumors that produce prolactin, with Bromocriptine or lisuride, has clinical relevance. (Thorner et al., 1980). Inhibition of prolactin secretion is mediated by stimulation of similar dopamine D2 receptor. However, the mode of action is completely different, he was responsible for the antitumor properties of certain Ergoline.

Using a cell of a mouse lymphoma L5178y has proven that festuclavina and agroclavina are cytostatic agents strong in vitro (Eich et al., 1984).

Their EC50 values of 6.3 μM, respectively 7.1 μM, were comparable in terms of effect force, with camptotecine EC50 value (7.2 μM), a cytostatic alkaloid used therapeutically.

Thus, festuclavine and agroclavine have unique biological activities as members of the ergot alkaloid family. This may be important not only for potential future drugs, but also for relevance of some aspects of chemical ecology (Eich, 1992).

For clavine development as potential anticancer agents, shall have regard four main objectives:1) Study of structure - activity relationship for discovery of compounds with enhanced *in vitro* cytotoxic potency.

2) Development of active compounds with enhanced metabolic stability, because ergolinele are rapidly metabolized in vivo.

3) Dissociation between antineoplasic and mutagenic activities, assuming that the second is not mechanism of action of the first.

4) Decrease affinity for neurotransmitters who mentioned above, on the assumption that the cytostatic effect is not based on interaction with any of these receptors.

In present, the compounds are tested for antineoplastic activity, and the most promising results seem to be from agroclavine ribosides.

## 1. MATERIALS AND METHODS

After conducting numerous tests on mice and rats *in vitro* was established LD50 dose of 50 μg/kg to be administered from product for to test. Because of its vasoconstrictor effect, it was been established intratumoral administration of gross fungal extract used.

Practical application in vivo has been established to be carried out on dogs and cats with mammary or rectal tumors.

The present study evaluated the effects of the product when were injected intratumoral in a rectal tumor of a dog and possible side effects that may the product determine, their duration, scale they may well and the possibility to reduce them.

This has been carried out biochemical tests to determine liver transaminases AST, ALT and GGT, determination of creatinine, urea, and other biochemical determinations, cholesterol, alkaline phosphatase, glucose, and also determine blood analysis by a specific protocol.

Product injection was performed once a week and tests were performed both before and after administration.

## 2. RESULTS AND DISCUSSION

The study was made with a gross extract of fungal strain T3-2 which after a week of fermentation have a composition of total alkaloid content (TAC) 3.61 mg / ml, 7.2 mg protein / ml and glucans 7 2 mg / ml.

The dose used was 50μg/kg body intratumoral administered once weekly. As a monitoring protocol was took the main constants of the animal in the study (temperature T, pulse P, respiration R) before to administration, after 24 hours, after 48 hours, after 72 hours and after 1 week.

Biochemical and hematological tests were performed: the first time before use, then at 24 hours after and after 1 week.

The study lasted 16 weeks ascertained the following:

***Table 1***

**Monitoring main constants from repeated administration of crude fungal extract T3-2 strain of Claviceps purpurea**

| Week | Ziua | Temp. $C^0$ | Pulse Bat/min | Respiration Resp/min |
|---|---|---|---|---|
| 1 I adm | 0 | 38.6 | 78 | 12 |
| | 1 | 38.5 | 75 | 13 |
| | 2 | 38.6 | 79 | 15 |
| | 3 | 38.9 | 83 | 19 |
| | 7 | 38.9 | 84 | 18 |
| 2 II adm | 1 | 39.2 | 84 | 16 |
| | 2 | 39.1 | 85 | 17 |
| | 3 | 39.2 | 82 | 15 |
| | 7 | 39.2 | 85 | 15 |
| 3 a III-a adm | 1 | 39.2 | 85 | 15 |
| | 2 | 39.1 | 82 | 13 |
| | 3 | 39.4 | 84 | 17 |
| | 7 | 39.4 | 85 | 17 |
| 7 a VII-a adm. | 1 | 39.7 | 110 | 22 |
| | 2 | 39.6 | 120 | 17 |
| | 3 | 39.8 | 131 | 29 |

| | | | | |
|---|---|---|---|---|
| | 7 | 39.8 | 133 | 28 |
| 9 a IX-a adm | 1 | 39.9 | 141 | 33 |
| | 2 | 39.6 | 126 | 28 |
| | 3 | 39.7 | 126 | 31 |
| | 7 | 39.6 | 121 | 23 |
| 13 a XIII-a adm | 1 | 39.7 | 137 | 31 |
| | 2 | 39.9 | 129 | 34 |
| | 3 | 39.9 | 121 | 34 |
| | 7 | 39.7 | 127 | 32 |
| 16 a XVI-a adm | 1 | 39.9 | 144 | 41 |
| | 2 | 39.8 | 131 | 32 |
| | 3 | 39.7 | 127 | 28 |
| | 7 | 39.7 | 127 | 29 |

Main constants were normal until the 3rd day after the III administration when changes were observed by increasing temperature is especially seen in a feverish state in which pulse and breathing remained constant.

We note that this feverish condition remained stable until the first day of the 7 week, when the temperature found was $39.7^0$ C and was detected a slight acceleration of pulse and even breathing too. In the 7th day of the week VII the changes was visible.

It also found higher increases of temperature on the first day of the ninth week, 2nd and 3rd day of the thirteenth week and the first day of the sixteenth week, and also it was found an increased heart rate and breathing over the permissible limits of the species.

These changes were due to the reaction that causes a fungal gross extract administered for antitumor role in the tumor, seen a necrotic tissue of tumor and even its exfoliation.

***Table 2.***

**Monitoring of biochemical changes following repeated administration of gross fungal extract T3-2 strain of Claviceps purpurea**

| Week | Day | AST | ALT | GGT | Creat | Uree | Chol | PA Pfosp alc | Glic |
|---|---|---|---|---|---|---|---|---|---|
| **Normal value** | 24 h | 8,9-49 ui/l | 8,2-57 ui/l | 1,0-9,7 ui/l | 0,5-1,6 mg/dl | 8,8-26 mg/dl | 116-254 mg/dl | 10,6-101 ui/l | 60-120 mg/dl |
| **1 I adm** | 0 | 28 | 29 | 7,2 | 0,9 | 8,9 | 259 | 130 | 78 |
| | 1 | 32 | 41 | 7,9 | 1,4 | 11,1 | 254 | 129 | 68 |
| | 7 | 46 | 49 | 9,1 | 1,8 | 19,9 | 261 | 131 | 91 |
| **2 a II-a adm** | 1 | 39 | 48 | 9,1 | 1,7 | 18,9 | 261 | 129 | 90 |
| | 7 | 48 | 51 | 9,0 | 1,9 | 28,8 | 282 | 188 | 81 |
| **3 a III-a adm** | 1 | 49.1 | 48.8 | 9.3 | 1.6 | 28.1 | 299 | 201 | 79 |
| | 7 | 61.2 | 72 | 10.1 | 2.1 | 31.1 | 291 | 251 | 76 |
| **7 a VII-a adm** | 1 | 65.5 | 70.1 | 9.7 | 2.4 | 31.1 | 298 | 272 | 80 |
| | 7 | 79.8 | 81.2 | 11.1 | 2.9 | 39.8 | 271 | 331 | 83 |
| **9 a IX-a adm** | 1 | 91.1 | 91.4 | 14.4 | 2.9 | 42.1 | 288 | 523 | 91 |
| | 7 | 98.7 | 99.1 | 14.8 | 2.9 | 52.1 | 271 | 581 | 92 |
| **13 a XIII-a adm** | 1 | 157 | 123.2 | 14.9 | 3.1 | 62.7 | 256 | 622 | 89 |
| | 7 | 182 | 151.1 | 16.1 | 3.7 | 65.9 | 267 | 634 | 79 |
| **16 a XVI-a adm** | 1 | 221 | 286.6 | 18.1 | 3.8 | 76.1 | 249 | 689 | 93 |
| | 7 | 281 | 291 | 18.1 | 4.1 | 76.1 | 255 | 701 | 91 |

In terms of biochemical changes were observed in most parameters monitored, less cholesterol and glucose that were maintained at an acceptable level throughout the experimental period. The most significant changes were found in measurements of liver transaminases (AST, ALT) and even the gammaglutamil transpherase leading to the values of AST 281 IU / l, ALT 291 IU / l and GGT 18.1 IU/ l.

Also have observed changes in creatinine and urea, reaching values after 16 weeks of 4.1 mg / dl, respectively 76.1 mg / dl.

There were significant changes in alkaline phosphatase seen its value reached after 16 weeks at 701 IU / l, which means an increase of seven times to normal.

The biochemical determinations were found elevated above normal from the third week of the maximum values of the sixteenth week.

These increases above normal were largely due to the toxic effect that it has fungus Claviceps purpurea on various organs but especially the liver and kidneys.

***Table 3***

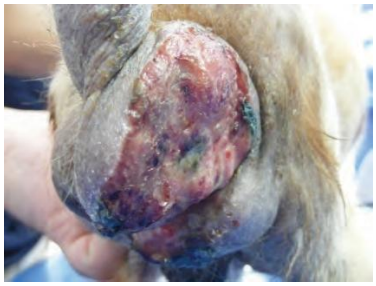
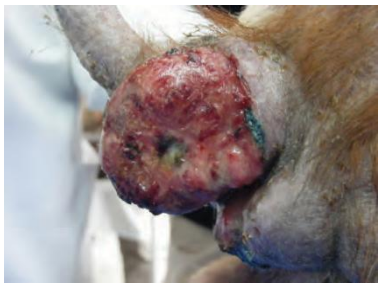
**Monitoring of hematological changes after repeated administration of gross extract of fungal strain of Claviceps purpurea T3-2**

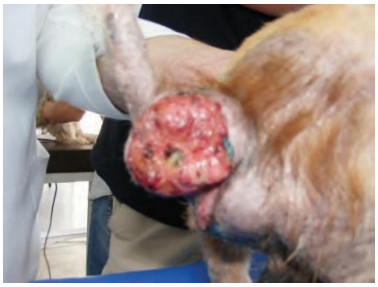
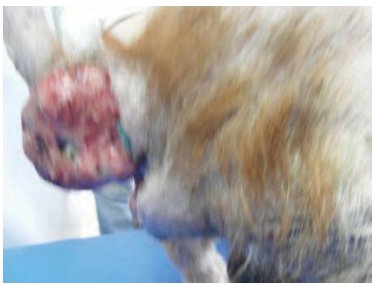
| Week | Day | WBC $X10^3/mm^3$ | RBC $X10^6/mm^3$ | HGB g/dl | HTC % | PLT $X10^3/mm^3$ | VEM $\mu m^3$ | HEM pg | CHEM g/dl | Limf % | Mon % | Gran % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Norm. Val. | 24 h | 6-17 | 5.40–7.80 | 13-19 | 37-54 | 160-430 | 64-74 | 22-27 | 34-36 | 12-30 | 3-10 | 62-83 |
| 1 I adm | 0 | 12 | 6.80 | 15.2 | 39.1 | 281 | 57.5 | 22.3 | 38.8 | 14.6 | 2.6 | 82.8 |
| | 1 | 12.6 | 7.01 | 14.8 | 39.6. | 293 | 56.5 | 21.1 | 37.4 | 11.6 | 2.3 | 86.1 |
| | 7 | 16.8 | 7.22 | 15.3 | 38.7 | 298 | 53.6 | 21.2 | 39.5 | 13.5 | 3.0 | 83.5 |
| 2 a II-a adm | 1 | 17.9 | 7.57 | 15.0 | 38.9 | 289 | 51.3 | 19.8 | 38.5 | 10.8 | 2.1 | 87.1 |
| | 7 | 16.8 | 7.91 | 14.9 | 38.8 | 281 | 49.0 | 18.8 | 38.4 | 9.0 | 2.1 | 88.9 |
| 3 a III-a adm | 1 | 17.1 | 7.80 | 14.7 | 38.6 | 279 | 49.4 | 18.8 | 38.1 | 10.4 | 2.0 | 87.6 |
| | 7 | 17.9 | 7.21 | 14.3 | 39.6 | 276 | 54.92 | 19.8 | 36.1 | 8.9 | 1.9 | 89.2 |
| 7 a VII-a adm | 1 | 19.7 | 6.2 | 12.9 | 42.1 | 288 | 67.9 | 20.8 | 30.6 | 7.3 | 1.4 | 91.3 |
| | 7 | 19.9 | 5.9 | 12.3 | 42.8 | 257 | 72.5 | 20.8 | 28.7 | 6.2 | 1.2 | 92.6 |
| 9 a IX-a adm | 1 | 18.9 | 5.9 | 12.1 | 44.0 | 261 | 74.5 | 20.5 | 27.5 | 9.3 | 1.6 | 89.1 |
| | 7 | 19.4 | 5.1 | 11.9 | 43.6 | 259 | 85.5 | 23.3 | 27.3 | 9.2 | 1.5 | 89.3 |
| 13 a XIII-a adm | 1 | 21.1 | 4.8 | 10.8 | 49.3 | 205 | 102.7 | 22.5 | 21.9 | 11.0 | 2.5 | 86.5 |
| | 7 | 21.3 | 4.9 | 10.8 | 48.9 | 217 | 99.8 | 22.0 | 22.1 | 8.6 | 2.3 | 89.1 |
| 16 a XVI-a adm | 1 | 20.7 | 4.5 | 10.9 | 49.8 | 201 | 110.6 | 24.2 | 21.9 | 9.1 | 1.9 | 89.0 |
| | 7 | 20.9 | 4.4 | 11.1 | 49.9 | 208 | 113.4 | 25.2 | 22.2 | 8.4 | 1.8 | 89.8 |

After monitoring blood was found that since three weeks the changes occurred in white series (WBC) by slight increase reaching values of 20.9 x103/mm$^3$ 16th week, changes in hemoglobin (HGB) by reducing its minimum values during the weeks of 13th and 16th. Haematocrit also increased significantly in lasts weeks, the highest increase being at 16 weeks but keeping the upper limit of the species. Due to changes in haematocrit, red blood cells and hemoglobin were found changes in erythrocyte constants derived MCV was found with maximum values at 16 weeks, minimum values HEM are in week 9 and minimum values of CHEM was being observed at weeks 13th and 16th. White series has the most significant changes in granulocyte level, changes in their upward from week 2 (87.1%) until the 16th week (89.8%).

With these changes we can see the body reacting to the toxic effects of fungal extracts, known since antiquity.

**Figure 1,2,3,4.**

**1.**  2. 

3.  4. 

The main effect followed was tumor volume reduction. Of the four images it is observed tumor reduction and clear delineation after 16 administration.

In Figure 1. Large rectal tumor that included the tail, was difficult surgical approach, with edema around and broad base of implantation.

In Figure 2. The apparent reduction of peritumoral edema and a more strict separation of it.

Figure 3. Shows reduction in volume of tumor tissue and its clear color.

Figure 4. Highlights the reduction in volume of tumor tissue and clear delineation of the tumor.

Since gross fungal extract side effects were obvious, was still it attempts by administration of drugs for life support after the 16-week study. It has been observed improvements of general condition and reduction of ALT, AST, creatinine, urea, and hematological reducing total WBC and granulocytes in particular.

Life support medication was performed by intravenous way of sodium chloride, glucose 5%, C vitamin and B1 and B6 vitamins, and if necessary were added Arginine sorbitol or Aspatofort.

Antibiotherapy was performed with Augmentin 15mg/kg for 2 weeks.

We believe that reducing the tumor in this case was a success.

Because it was only the first in vitro study in the research contract with this theme we hope to purify the extract for alleviate possible adverse effects, or perfusion for life support throughout the experiment.

However we can say that the side effects of fungal gross extract used, were not stronger than the side effects of chemotherapy agents referred to in the literature.

## 3. CONCLUSIONS

3.1. Gross fungal extract was used for its vasoconstrictor effect pursued the reduction of external tumors (rectal or breast) with difficult surgical approach.

3.2. After 16 repeated once in a week, it was observed a decrease in the volume of tumor tissue, reduce peritumoral edema and clear delineation of tumor from healthy tissue.

3.3. Side effects of gross fungal extract in tumor administered was seen especially in the liver and kidney by significant increases of most importants parameters of liver and kidney.

3.4. Side effects of fungic gross extract administered in tumor could be attenuated following a general supportive treatment by powerful antibiotics with broad spectrum and administering a more liver protective.

## ACKNOWLEDGEMENTS

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# STUDY CONCERNING THE PHYSICO-CHEMICAL CHARACTERISTICS OF RECONSTITUTED COW MILK IN COMPARISON TO COW MILK

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**Key words**: milk powder, physicochemical characteristics, reconstitution, comparison.

**SUMMARY**

A number of samples of reconstituted milk and usual cow milk were analyzed and results were evaluated in order to determine the possible differences between the two types of milk. Data showed, as an overall image, no differences between the two products. However, values considering density, lactose percent and protein content were higher in reconstituted milk, in comparison to usual pasteurized cow milk. Another very interesting point was that the solids-non fat content was higher in reconstituted milk in comparison to cow milk, possibly due to the enrichment of milk powder in mineral substances and other nutrients.

Powdered milk is frequently used in the manufacture of infant formula, confectionery such as chocolate and caramel candy, and in recipes for baked goods where adding liquid milk would render the product too thin. Milk powders contain all twenty standard amino acids (the building blocks of proteins) and are high in soluble vitamins and minerals (Lei et al., 2010).

Milk powder manufacture involves the gentle removal of water at the lowest possible cost under stringent hygiene conditions while retaining all the desirable natural properties of the milk-color, flavor, solubility, nutritional value. Whole (full cream) milk contains, typically, about 87% water and skim milk contains about 91% water (Baldwin, 2007).

During milk powder manufacture, this water is removed by boiling the milk under reduced pressure at low temperature in a process known as evaporation. The resulting concentrated milk is then sprayed in a fine mist into hot air to remove further moisture and so give a powder. Approximately 13 kg of whole milk powder (WMP) or 9 kg of skim milk powder (SMP) can be made from 100 L of whole milk (Fitzpatrick et al., 2004; Mimouni et al., 2009). The purpose of this paper was to evaluate the physicochemical properties of reconstituted milk in comparison to the ones that characterize cow milk, and to detect any difference in their composition.

## 1. MATERIALS AND METHODS

A series of samples of powder milk have been collected from a local market and reconstituted in the laboratory. In parallel, another batch of samples comprising pasteurized cow milk has been purchased from a local store. Both types of samples were let at room temperature and analyzed using EKOMILK TOTAL. The analyzed parameters are included in table 1.

***Table 1***

**The measuring parameters for EKOMILK TOTAL**

| | |
|---|---|
| **Fatness (F)** | 0,5% to 12% with accuracy ± 0,1% |
| **Solids non fat (SNF)** | 6% - 12% with accuracy ± 0.2% |
| **Milk density (D)** | 1,0260 g/cm³ - 1,0330 g/cm³± 0.0005 g/cm³ |
| **Protein (P)** | 2% - 6% with accuracy ± 0.2% |
| **Lactose (L)** | 0.5% to 7% with accuracy ± 0,2% |
| **Freezing point (FP)** | from 0 to -1.000 °C |
| **Added water to milk (AWM)** | 0% - 60% with accuracy ± 5% |
| **pH** | 0,00 – 14 pH with accuracy ± 0,02 |
| **Conductivity (C)** | 2 mS/cm - 20 mS/cm ± 0.5 % (18°C) |
| **Temperature (T)** | 0 - 50 °C with accuracy± 0,1 °C |

## 2. RESULTS AND DISCUSSIONS

A total number of 60 samples per each milk category was analyzed, and data was calculated per each batch of samples. Dried milk was analyzed per category, concerning the percent of fat, type 26 (26 %, per dried mass), type 20 (20 %), and skimmed type (1,5 %). Cow milk was also classified by fat percent, in: whole milk (3,5 %), skimmed milk (1,5 %), and diet milk (0,1 %). A number of 20 samples was analyzed for each category, for cow milk as for dried milk also.

The results for all types of dried milk and cow milk are showed in table 2 and 3, as average values.

***Table 2***

**Average values of the main physicochemical parameters of dried reconstituted milk analyzed with EKOMILK TOTAL**

| Milk type | F | SNF | D | P | L | FP | AWM | pH | Z | T |
|---|---|---|---|---|---|---|---|---|---|---|
| **Type 26** | 3,49 | 11,87 | 30,8 | 5,97 | 5,10 | 56,50 | 0,00 | 6,86 | 4,21 | 19,10 |
| **Type 20** | 2,98 | 12,41 | 29,9 | 5,95 | 5,65 | 58,90 | 0,00 | 6,58 | 4,58 | 19,50 |
| **Skimmed type** | 1,12 | 11,86 | 29,4 | 5,78 | 5,28 | 59,10 | 0,00 | 6,64 | 4,47 | 18,90 |

Results show higher values for type 26 than for the others concerning fat percent, density, amount of protein, pH, but the lowest percent of lactose, value of conductivity and freezing point.

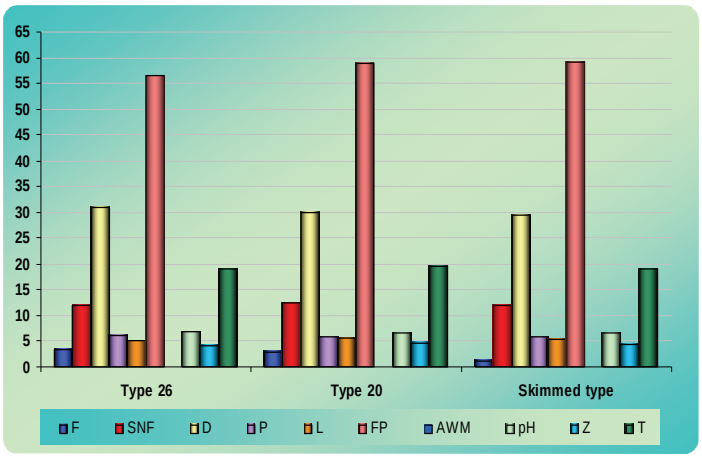


**Fig. 2 - Average values of the main physicochemical parameters of dried reconstituted milk analyzed with EKOMILK TOTAL**

Type 20 presents a medium amount of fat and protein, a medium value for density and freezing point, the highest conductivity value and also the highest amount of solids non fat, in comparison to the other two types of powder milk.

Skimmed reconstituted milk had a 1,12 % fat, a density of 1,0294 g/cm$^3$, a low content in protein (5,78 %), a medium percent for lactose (5,28 %), the highest freezing point, -0,591$^o$C, in comparison to type 26 with -0,565$^o$C and type 20 with -0,589$^o$C, the lowest pH and a medium value for conductivity, 4,47 mS/cm.

In all cases, the percent of added water (AWM) was 0, meaning that water used for reconstitution solubilized all components in an optimum way and the reconstituted milk is very similar as properties to usual cow milk.

***Table 3***

**Average values of the main physicochemical parameters of cow milk analyzed with EKOMILK TOTAL**

| Milk type | F | SNF | D | P | L | FP | AWM | pH | Z | T |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Whole*** | 3,51 | 9,03 | 29,20 | 3,55 | 4,68 | 59,40 | 0,00 | 6,76 | 4,84 | 18,70 |
| ***Skimmed*** | 1,57 | 8,91 | 28,80 | 3,28 | 4,82 | 56,80 | 0,00 | 6,58 | 4,75 | 18,50 |
| ***Diet*** | 0,12 | 8,76 | 28,70 | 3,39 | 4,57 | 57,30 | 0,00 | 6,67 | 4,69 | 18,90 |

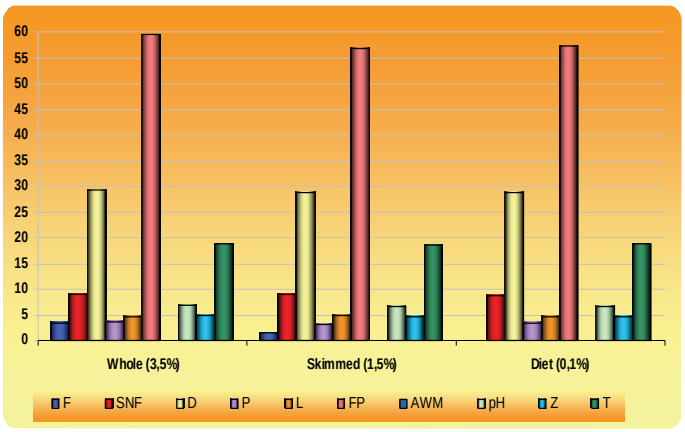


**Fig. 3 - Average values of the main physicochemical parameters of usual cow milk analyzed with EKOMILK TOTAL**

Concerning cow milk, fat percentage was adequate for the type of commercial milk. No added water was detected. The highest percentage of solids non-fat was observed in whole milk, 9,03 %, and the lowest in diet milk, only 8,76 %.

The highest amount of protein was observed in whole milk, 3,55 % and the lowest in skimmed milk, only 3,28 %. Skimmed milk had the highest amount of glucose (4,82 %), while diet milk the lowest (only 4,57 %). The highest freezing point value was observed in whole milk, and the same category of milk had also the highest pH and conductivity, while diet milk had the lowest. Skimmed milk presented medium values concerning these parameters. As a comparison, data for dried reconstituted milk and those for usual cow milk are displayed together in table 4, in order to summarize the main differences. As the table and the figure presented, the main physicochemical parameters for both classes of milk do not differ in large scale. However, it seems that, overall, density is lower in cow milk, in comparison to the one for reconstituted milk, as well as lactose percent (the difference in very small) and protein content. The pH was constant, with close values for both classes of milk. A very interesting point is that SNF (solids-non fat), a parameters that evaluates the quantity of solid substance other than fat, presented higher values in reconstituted milk, in comparison to usual cow milk.

*Table 4 –*

**Comparison between the main physicochemical parameters for reconstituted milk and usual cow milk**

| Type / Parameters | *Reconstituted milk* | | | *Usual cow milk* | | |
|---|---|---|---|---|---|---|
| | **26** | **20** | **Skimmed** | **Whole** | **Skimmed** | **Diet** |
| **F** | 3,49 | 2,98 | 1,12 | 3,51 | 1,57 | 0,12 |
| **SNF** | 11,87 | 12,41 | 11,86 | 9,03 | 8,91 | 8,76 |
| **D** | 30,8 | 29,9 | 29,4 | 29,20 | 28,80 | 28,70 |
| **P** | 5,97 | 5,95 | 5,78 | 3,55 | 3,28 | 3,39 |
| **L** | 5,10 | 5,65 | 5,28 | 4,68 | 4,82 | 4,57 |
| **FP** | 56,50 | 58,90 | 59,10 | 59,40 | 56,80 | 57,30 |
| **pH** | 6,86 | 6,58 | 6,64 | 6,76 | 6,58 | 6,67 |
| **Z** | 4,21 | 4,58 | 4,47 | 4,84 | 4,75 | 4,69 |
| **T** | 19,10 | 19,50 | 18,90 | 18,70 | 18,50 | 18,90 |

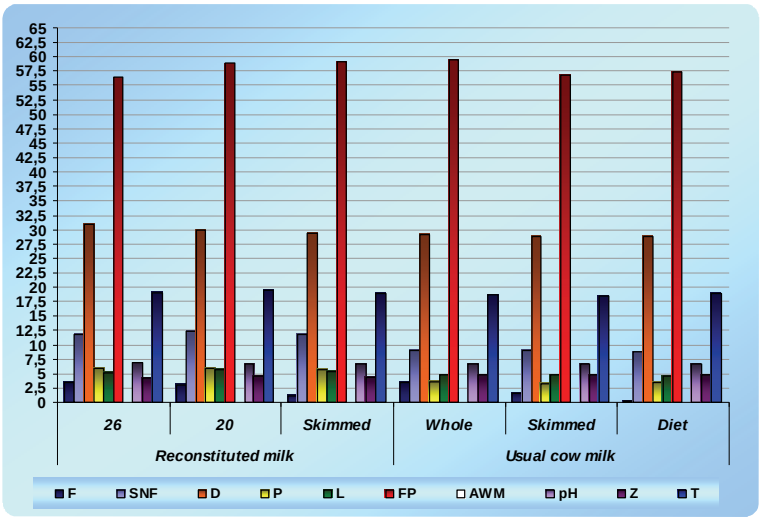


**Fig. 4. - Comparison between the main physicochemical parameters for reconstituted milk and usual cow milk**

The reason for this specific characteristic is that milk powder, a product destined to use for babies in the first year of life, is enriched in mineral substances and other nutrients.

## 3. CONCLUSIONS

3.1. The reconstituted milk is a very important type of milk for industry and also as a baby food, containing a series of nutritive

substances very close to what the human organism needs, with a complete content in amino acids and minerals.

3.2. Overall, reconstituted milk showed similar physicochemical parameters in comparison to usual pasteurized cow milk, sold in local markets.

3.3. A slight difference was observed considering density, lactose percent and amount of protein (all values higher in reconstituted milk).

3.4. A very interesting point is that solids-non fat content was quite higher in reconstituted milk, in comparison to usual cow milk, potentially due to the substances used for the enrichment of this commercial milk product.

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# COMPARISON BETWEEN SOME PHYSICOCHEMICAL PARAMETERS OF SEVERAL TYPES OF MILK OBTAINED FROM DIFFERENT SPECIES – COW, GOAT AND SHEEP REARED IN THE PROXIMITY OF BUCHAREST METROPOLITAN AREA

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**Key words:** cow, goat, sheep, milk, characteristics

**SUMMARY**

The physicochemical characteristics of milk are in strict correlation with the composition for a particular animal species. For example, milk obtained from sheep presents higher level of solid non fat substances, in comparison with goat and cow milk, and the total quantity of nutritive substances is also higher. A number of 960 samples of cow, goat and sheep milk were collected and analyzed concerning physicochemical parameters, an mean values were calculated. The period of the study was of 8 months, from February until September, and weekly a number of 10 samples were collected, transported to the laboratory and analyzed. After data was processed and graphics were elaborated, a final comparison was made concerning the conformity with the standards and the physicochemical traits of each type of milk.

Dairy goat, dairy sheep and dairy cow farming represent a very important part of the economy in many countries in the EU, although large-scale industrialization of the dairy goat and dairy sheep sectors is still limited by several factors (Haenlein, 2006). There are a lot of differences between the physicochemical properties of milk from the main production species, and this is precious information concerning economic status of the field and also for the consumer (Park, 2006). Cow milk presents only minimal changes during the seasons in a year, but sheep and goat milk is obtained by seasonal breeding of ewes and does, therefore changes in the composition of milk may occur frequently. Goat milk presents a very important trait in comparison to cow milk, being more alkaline, having a buffering capacity and a higher digestibility (Hui, 2007). Viscosity, gravity, refractive index and titratable acidity is higher in sheep milk, than in cow milk, and the freezing point is lower. The composition of goat, sheep and cow milk vary with the diet, breed, individuals, parity, season, feeding, management, environmental conditions, stage of lactation and health status of the udder (Remeuf, 2008). The purpose of this paper is to evaluate the characteristics of

cow, sheep and goat milk in comparison to one another, as a form of assessment considering biochemical traits.

## 1. Materials and methods

A number of 10 samples of milk, for each species, was collected weekly, for a period of 8 months, and stored in refrigeration conditions, in order to maintain its properties until analysis in laboratory, the same day. The entire period of the study was between February and September 2010, and the samples were collected weekly and analyzed the same day, in order to obtain precise results. The samples of cow milk were selected from a local breeding unit near Bucharest, while samples of milk from sheep and goat were collected from local producers. The device used for physicochemical analysis of milk is EKOMILK TOTAL, an automated multi-parameter milk analyzer providing rapid test results for: Fat, Protein, Solids Not Fat, Lactose, Density, Freezing point, Added Water, pH, Temperature and Conductivity in fresh milk (cow, sheep and/or buffalo, goat). The measuring parameters are listed in table 1.

***Table 1 –***

**The measuring parameters for EKOMILK TOTAL**

| | |
|---|---|
| **Fatness (F)** | 0,5% to 12% with accuracy ± 0,1% |
| **Solids non fat (SNF)** | 6% - 12% with accuracy ± 0.2% |
| **Milk density (D)** | 1,0260 g/cm³ - 1,0330 g/cm³± 0.0005 g/cm³ |
| **Protein (P)** | 2% - 6% with accuracy ± 0.2% |
| **Lactose (L)** | 0.5% to 7% with accuracy ± 0,2% |
| **Freezing point (FP)** | from 0 to -1.000 °C |
| **Added water to milk (AWM)** | 0% - 60% with accuracy ± 5% |
| **pH** | 0,00 – 14 pH with accuracy ± 0,02 |
| **Conductivity (C)** | 2 mS/cm - 20 mS/cm ± 0.5 % (18°C) |
| **Temperature (T)** | 0 - 50 °C with accuracy± 0,1 °C |

## 2. RESULTS AND DISCUSSIONS

With a total number 320 milk samples/8 months, for each species, (960 milk samples, the total number), we recorded the data and calculated the average values per each month and per entire period of the study. The results (average values per month and year) for each milk type (per species) are listed in tables 2, 3 and 4, and presented as mean values per entire study period in figures 1, 2 and 3.

***Table 2 –***

**Physicochemical characteristics of cow milk during the months of February and September (average values)**

| Month | F | SNF | D | P | L | FP | AWM | pH | Z | T |
|---|---|---|---|---|---|---|---|---|---|---|
| *Feb.* | 3,65 | 8,16 | 30,40 | 3,09 | 4,75 | 55,50 | 0,00 | 6,79 | 4,24 | 18,70 |
| *Mar.* | 3,52 | 8,29 | 29,50 | 3,16 | 5,02 | 56,90 | 0,00 | 6,81 | 4,48 | 18,40 |
| *Apr.* | 3,45 | 8,38 | 29,30 | 3,18 | 5,08 | 60,50 | 0,00 | 6,70 | 4,21 | 18,30 |
| *May* | 3,62 | 9,36 | 27,80 | 3,53 | 4,95 | 55,40 | 0,00 | 6,76 | 4,75 | 18,70 |
| *Jun.* | 3,68 | 9,29 | 26,80 | 3,50 | 4,58 | 58,40 | 0,00 | 6,75 | 4,69 | 19,00 |
| *Jul.* | 3,64 | 8,44 | 27,40 | 3,18 | 4,91 | 55,90 | 0,00 | 6,12 | 4,71 | 18,60 |
| *Aug.* | 3,59 | 8,75 | 28,60 | 3,31 | 4,60 | 56,20 | 0,00 | 6,45 | 4,86 | 18,90 |
| *Sept.* | 3,54 | 9,17 | 31,00 | 3,45 | 5,06 | 59,60 | 0,00 | 6,18 | 4,84 | 18,80 |
| *Mean value* | *3,58* | *8,73* | *28,85* | *3,30* | *4,86* | *57,30* | *0,00* | *6,57* | *4,59* | *18,65* |

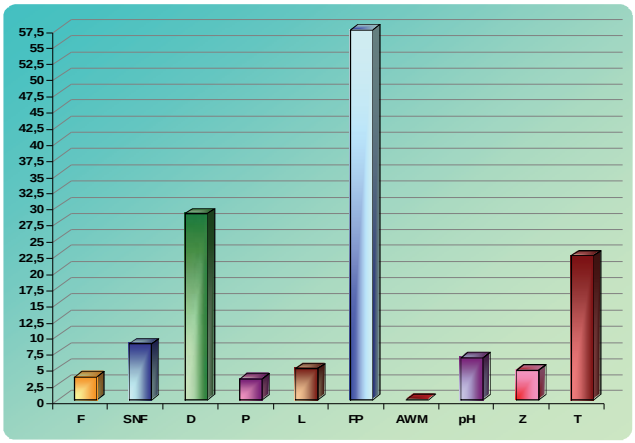


**Fig. 1 - Physicochemical characteristics of cow milk (average values) for the entire month of study.**

***Table 3 –***

**Physicochemical characteristics of goat milk during the months of February and September (average values)**

| Month | F | SNF | D | P | L | FP | AWM | pH | Z | T |
|---|---|---|---|---|---|---|---|---|---|---|
| *Feb.* | 3,58 | 8,33 | 29,50 | 3,12 | 4,41 | 56,10 | 0,00 | 6,49 | 3,58 | 18,70 |
| *Mar.* | 3,61 | 8,57 | 29,60 | 3,38 | 4,39 | 55,50 | 0,00 | 6,52 | 3,69 | 18,50 |
| *Apr.* | 3,68 | 8,47 | 30,10 | 3,29 | 4,38 | 55,10 | 0,00 | 6,55 | 3,48 | 18,00 |
| *May* | 3,69 | 8,45 | 30,20 | 3,25 | 4,40 | 54,90 | 0,00 | 6,48 | 3,53 | 18,10 |
| *Jun.* | 3,73 | 8,34 | 29,80 | 3,17 | 4,37 | 54,80 | 0,00 | 6,42 | 3,48 | 18,80 |
| *Jul.* | 3,71 | 8,32 | 29,80 | 3,21 | 4,31 | 55,10 | 0,00 | 6,45 | 3,74 | 18,50 |
| *Aug.* | 3,72 | 8,59 | 29,90 | 3,34 | 4,45 | 55,90 | 0,00 | 6,50 | 3,26 | 18,50 |
| *Sept.* | 3,79 | 8,40 | 29,70 | 3,18 | 4,42 | 55,70 | 0,00 | 6,44 | 3,31 | 18,20 |
| *Mean value* | *3,68* | *8,44* | *29,80* | *3,24* | *4,39* | *55,40* | *0,00* | *6,48* | *3,51* | *18,41* |

The mean value – considering cow milk – for fat content was 3,58, the total SNF (solids-non fat) was of 8,73, the density had ranges between 1,026 and 1,031, with a mean value of 1,028 g/cm³, the percent of protein was 3,3, lactose was present in a total of 4,86 %, the freezing point had values of -0,573°C. The pH of cow milk had an acid value of 6,57, while conductivity presented a mean value of 4,59 mS/cm. Goat milk presents results within ranges for quality milk: 3,68 % fat, 3,24 % protein, 4,39 % lactose, a conductivity of 3,51mS/cm, a pH of 6,48 (slightly acid), a freezing point value of -0,554°C, a density of 1,029 g/cm$^3$ and a content of solids non fat of 8,44 %.

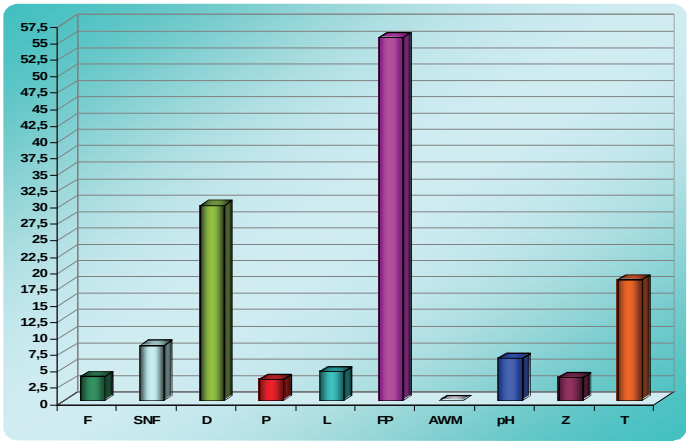


**Fig. 2 - Physicochemical characteristics of goat milk (average values) for the entire month of study.**

***Table 4***

**Physicochemical characteristics of sheep milk during the months of February and September (average values)**

| Month | F | SNF | D | P | L | FP | AWM | pH | Z | T |
|---|---|---|---|---|---|---|---|---|---|---|
| *Feb.* | 6,12 | 11,31 | 27,80 | 5,41 | 5,13 | 55,40 | 0,00 | 6,47 | 3,31 | 18,80 |
| *Mar.* | 6,03 | 11,28 | 29,40 | 5,39 | 5,09 | 54,80 | 0,00 | 6,48 | 3,56 | 18,60 |
| *Apr.* | 6,08 | 11,21 | 31,80 | 5,38 | 5,02 | 54,90 | 0,00 | 6,52 | 3,78 | 18,40 |
| *May* | 5,94 | 11,15 | 31,20 | 5,35 | 5,00 | 55,10 | 0,00 | 6,55 | 3,46 | 18,80 |
| *Jun.* | 5,87 | 11,28 | 28,50 | 5,37 | 5,11 | 55,80 | 0,00 | 6,50 | 3,65 | 18,50 |
| *Jul.* | 5,96 | 11,19 | 30,10 | 5,29 | 5,10 | 56,20 | 0,00 | 6,61 | 3,38 | 18,30 |
| *Aug.* | 5,99 | 11,18 | 31,00 | 5,40 | 4,98 | 54,90 | 0,00 | 6,67 | 3,41 | 18,00 |
| *Sept.* | 6,02 | 11,31 | 32,00 | 5,34 | 5,17 | 55,50 | 0,00 | 6,60 | 3,52 | 18,40 |
| *Mean value* | *6,00* | *11,23* | *30,20* | *5,36* | *5,07* | *55,30* | *0,00* | *6,55* | *3,51* | *18,50* |

Sheep milk is also in accordance with the standard values, with: 6 % fat, 5,36 % protein, 5,07 % lactose, a density of 1,030 g/cm$^3$ and a solids non fat content of 11,23 % (normal for this type of milk). It also

presented values of 6,55 for pH, a conductivity of 3,51 mS/cm and a freezing point of -0,553°C.

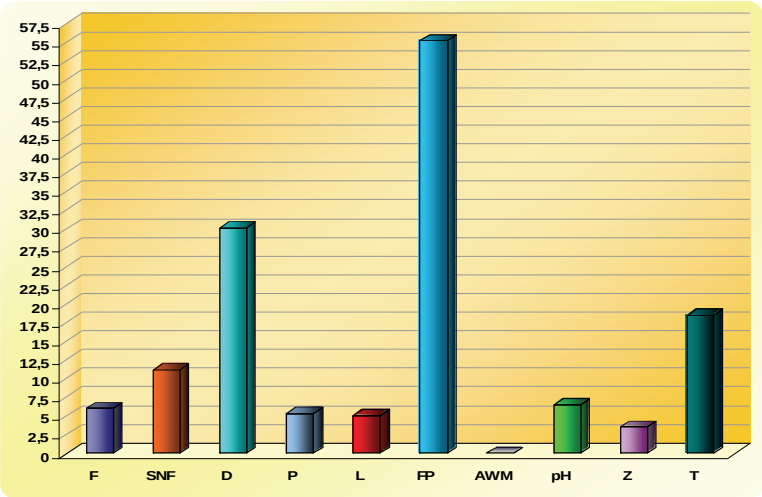


**Fig. 3 - Physicochemical characteristics of sheep milk (average values) for the entire month of study.**

***Table 5***

**Comparison between the mean values resulted for each type of milk taking into account the species**

| | F | SNF | D | P | L | FP | pH | Z |
|---|---|---|---|---|---|---|---|---|
| **Cow milk** | *3,58* | *8,73* | *28,85* | *3,3* | *4,86* | *57,3* | *6,57* | *4,59* |
| **Goat milk** | *3,68* | *8,44* | *29,8* | *3,24* | *4,39* | *55,4* | *6,48* | *3,51* |
| **Sheep milk** | *6* | *11,23* | *30,2* | *5,36* | *5,07* | *55,3* | *6,55* | *3,51* |

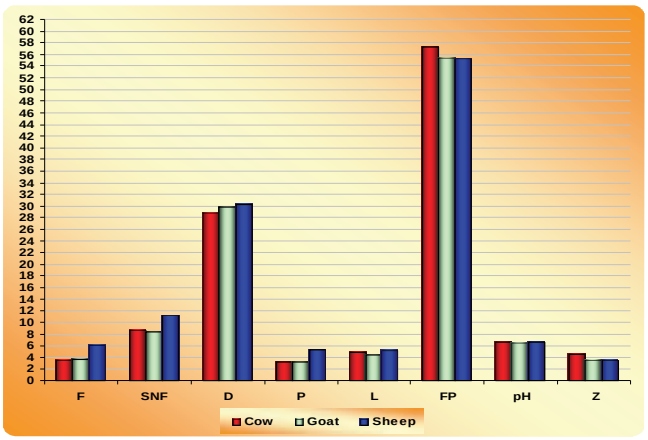


**Fig. 4 -** Comparison between the mean values resulted for each type of milk taking into account the species

Considering the fat content, sheep milk had the highest percentage, 6 %, in comparison to goat milk, with 3, 68 % and cow milk, with the

lowest, 3,58 %. SNF percentage was also highest concerning sheep milk, 11,23 %, in comparison to 8,73 % for cow milk and only 8,44 % for goat milk. The highest density was registered for sheep milk, with 1,030 g/cm$^3$, while goat milk had an average value of 1,029 g/cm$^3$ and cow milk 1,028 g/cm$^3$. Protein level is 5,36 % in sheep milk, higher than in cow milk, 3,3 % and goat milk 3,24%. The percentage of lactose was also the highest in sheep milk, 5,07 %, while cow milk had 4,86 % and goat milk only 4,39 %. The highest pH was registered by cow milk, 6,57, followed by sheep milk, with 6,55 and 6,48 for goat milk. The highest freezing point was observed for sheep milk, -0,553$^o$C, while goat milk presented a value of -0,554$^o$C and cow milk, -0,573$^o$C. The conductivity was highest for cow milk, 4,59 mS/cm, and equal as value for both goat and sheep milk, 3,51 mS/cm.

## 3. CONCLUSIONS

3.1. Sheep milk remains the richest milk in fat, protein, lactose and other constituents, while cow milk presented the highest pH and electrical conductivity.

3.2. Cow milk presents, in comparison to the other two types studied in this survey, average values, being higher in protein, lactose and solids-non fat than goat milk, the latter having a higher percentage of fat.

3.3. All the values obtained during this study were situated in range, considering the standard and legislation that apply in this sector of the food industry.

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# PRELIMINARY DATA ON FIRST MOLECULAR SCREENING FOR PATHOGENS OF TICKS IN ROMANIA

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**Key words**: ticks, pathogens, PCR, screening

## SUMMARY

The prevalence of tick-borne pathogens (*Babesia, Theileria, Rickettsia* species) in questing and feeding adult ticks was determined for first time in Romania. A total of 165 ticks were analyzed in the study. Of these, 40 were questing ticks and 125 were collected from naturally infested cattle, sheep or goats. Tick species analyzed included *Ixodes ricinus, Dermacentor marginatus*, *Rhipicephalus bursa* and *Hyalomma plumbeum plumbeum*.

In one of the total 40 questing *Hyalomma* specimens analyzed (representing 2.5%), *Babesia/Theileria equi* was detected, confirming the role of *Hyalomma* ticks as vector in transmission of horse piroplasmosis. Of the feeding adult ticks analyzed, 10.48% were found infected with different pathogens: *Babesia* spp. was detected in 3.99% of *Hyalomma plumbeum*, *Theileria* spp. was detected in 6.42% of *Hyalomma plumbeum* and in 33.33% of *Ixodes ricinus; Rickettsia* spp. was detected in 16.6% of *Dermacentor marginatus*.

The results of the present study emphasize differences in the role that different tick species play in the pathogen's life cycle and represent a base for further epidemiological studies of tick-borne diseases in Romania.

Ticks are considered, after mosquitoes, the most important vectors for infectious diseases worldwide (Parola and Raoult, 2001). They transmit a greater variety of pathogenic microorganisms (protozoa, rickettsiae, spirochaetes and viruses) than any other arthropod vector group, and a significant number of these pathogens are agents of emerging infectious diseases (Jongegan and Uilenberg, 2004).

The development and implementation of control measures for tick-borne pathogens is dependent on understanding the epidemiology of these pathogens in a particular geographical region (Gray et al., 2009).

Romania is a very diverse country with many different habitats, even within a relatively small area, and different climates and flora across the country are found. In the last years, several studies on eco-biology and seasonal dynamics of tick species in Romania have reported an increased tick populations abundance (Mitrea and Ionita, 2004; Ionita et al., 2006, 2009), and the risks of tick-borne diseases are likely to increase as well.

In this study, we conducted a preliminary molecular screening of ticks for emphasizing the prevalence of *Babesia, Theileria* and *Rickettsia* species in questing and feeding adult ticks in Romania.

## 1. MATERIALS AND METHODS

*Tick sampling.* A total of 165 ticks were analyzed in this study. Of these, 40 were questing ticks and 125 were collected from naturally infested animal hosts (cattle, sheep or goats). Ticks were collected during of June - July period of the year 2010.

Host-seeking adult Ixodidae ticks were collected from vegetation, using the flag's method. Briefly, a 2x1.6 blanket was dragged for 20-30 min, stopping every 2-5 min to collect all adult ticks attached to the blanket. The distance covered in 5 min ranged between 100 m and 150 m depending on the slope of the terrain and density of scrub. Sampling was carried out in a forest area from Valcea county (in the South of Romania), bordering on pastures that are frequented by livestock.

Ticks were introduced in 70% ethanol and conserved at 4°C until taxonomic identification (based on morphological keys).

*DNA extraction*

Ticks were individually crushed and DNA was extracted using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. A negative control was added at the beginning and at the end of each extraction line. Quality and quantity of extracted DNA were evaluated in a spectrophotometer (NanoDrop ND-1000, PeqLab, Erlangen, Germany).

*PCR amplification and sequencing*

**Polymerase Chain Reaction for detection of *Rickettsia* spp**. The PCRs were performed under conditions published previously (Regnery et al. 1991, Silaghi et al., 2008) and targeted a 380-bp portion of the *gltA* gene by using the primer pair RpCS.877p/RpCS.1258n, and a 530-bp portion of the *ompA* gene by using the primer pair Rr190.70p/Rr190.602n (Regnery et al. 1991). The final PCR products were analyzed by 1.5% agarose gel-electrophoresis.

**The PCR reactions for the detection of *Babesia/Theileria* spp.** were performed as previously described (Casati et al., 2006) and targeted the common sequences 18S rRNA gene using primer BJ1 and BN2.

**DNA sequencing**. The PCR products were sequenced after purification with the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany), and the sequence homology searches were made by BLAST analysis of GenBank.

## 2. RESULTS AND DISCUSSIONS

The prevalence of *Babesia, Theileria* and *Rickettsia* species was analyzed in questing and feeding adult ticks from South of Romania. A total of 165 adult ixodid ticks were collected and analyzed in this study. Tick species analyzed included *Ixodes ricinus, Dermacentor marginatus*, *Rhipicephalus bursa* and *Hyalomma plumbeum plumbeum* (Table 1).

***Table 1***

**Tick species included in the study**

| Tick species | Questing ticks | | Feeding ticks sorted by host | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | | | sheep | | goats | | cattle | | |
| | male | female | male | female | male | female | male | female | |
| *Ixodes ricinus* | - | - | - | 2 | - | - | - | 1 | **3** |
| *Dermacentor marginatus* | - | - | 7 | 5 | - | - | - | - | **12** |
| *Rhipicephalus bursa* | 11 | 3 | - | - | - | 1 | - | - | **15** |
| *Hyalomma plumbeum plumbeum* | 18 | 8 | 34 | 21 | 7 | - | 12 | 35 | **135** |
| **Total** | **29** | **11** | **41** | **28** | **7** | **1** | **12** | **36** | **165** |
| | **40** | | **69** | | **8** | | **48** | | |

Of the all 40 *Hyalomma* questing tick specimens analyzed, one was found infected with *Babesia equi* (2.5% in prevalence).

Within the total feeding adult ticks, 10.48% were found infected with different pathogens (Table 2), the infected ticks belonging to all species involved in the study, with the exception of *Rhipicephalus bursa*. However, only a limited number of the last species was analyzed.

From the feeding *Hyalomma plumbeum* ticks, 3.99% were positive for *Babesia* spp. DNA, and 6.42% were positive for infection with *Theileria* spp. No mixed infections with both pathogens were detected. Of the *Babesia* positive specimens, two were fed on sheep and one on cattle, while the *Theileria* positive ticks, three feed on sheep and four on cattle.

*Dermacentor marginatus* was found infected with *Rickettsia* spp., with 16.6% in prevalence, the infected ticks being collected from sheep.

Although only three specimens of *Ixodes ricinus*, feeding on sheep, were included in the study, one was positive for *Theileria* spp., having 33.3% in prevalence.

*Table 2*

**Prevalence of tick-borne pathogens in feeding adult ixodid ticks in Romania**

| Pathogen species | Tick species | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Hyalomma plumbeum plumbeum* | | *Dermacentor marginatus* | | *Ixodes ricinus* | | *Rhipicephalus bursa* | |
| | n | % | n | % | n | % | n | % |
| *Babesia spp.* | 3/109 | *3.99* | - | - | - | - | - | - |
| *Theileria spp.* | 7/109 | *6.42* | - | - | 1/3 | *33.33* | - | - |
| *Rickettsia* spp. | - | - | 2/12 | *16.6* | - | - | - | - |

For all positive samples obtained, the products amplified in the PCRs were subjected to **DNA sequencing**, and the sequence homology searches were made by BLASTn analysis of GenBank. Sequences were subjected to National Center for Biotechnology Information (NCBI) BLAST analysis for the homology.

Therefore, for the *Babesia* spp. detected in one questing *Hyalomma plumebum plumebum*, NCBI BLAST analysis revealed 99% homology with *Babesia equi*. This current findings confirmed the role of *Hyalomma* in transmission of horse piroplasmosis; species of *Hyalomma* genera have beeng reported by many authors as vector for *Babesia caballi* or *Babesia (Theileria) equi* (Badescu, 1969; Feider, 1965; Pomerantzev, …; Mehlhorn and Schein, 1998), Estrada at al., 2004).

In cases of the positive *Theileria* samples of the feeding ticks, NCBI BLAST analysis revealed 99% homology with *Theileria buffeli, T. sergenti* and *T. orientalis*. All those three *Theileria* species, which are defined by some authors as *Theileria orientalis* group (Gubbels et al., 1999), are causing theileriosis in cattle, but usually benign forms are reported (Gubbels et al., 1999; Garcia-Sanmartin et al., 2006). However, this does not exclude the possibility that subclinical infections with *T. buffeli* might cause a production decrease (Garcia-Sanmartin et al., 2006). Whereas the vector of *T. buffeli* or *T. sergenti* are reported *Haemaphysalis* spp., *Dermacentor* or *Ixodes ricinus*, further research is needed to establish also the role of *Hyalomma* species in the epidemiology of cattle theileriosis, in particular in Romania.

Three feeding ticks of *Hyalomma plumbeum* were found infected with *Babesia* spp. For those samples, NCBI BLAST analysis revealed 97% homology with *Babesia ocultans*, but also 98% homology with *Babesia bigemina*.

*Babesia occultans*, first isolated from engorged adult female ticks of *Hyalomma marginatum rufipes* (Thomas and Mason, 1981), is the causative agent of a benign form of cattle babesiosis in South Africa. It causes a mild disease in cattle and is transmitted transovarially by *Hyalomma marginatum rufipes* (Thomas and Mason, 1981; Gray and De Vos, 1981). Since this species occurred at low numbers and did not seem to cause significant clinical reactions in normal animals, the specific name *Babesia occultans* sp. nov. was proposed (Gray and De Vos, 1981).

The *B. occultans* 18S rRNA gene sequence differed by only one base pair from the sequences of the unnamed *Babesia* species (U09834) and *Babesia* sp. Kashi 2. While it has been reported that these parasites are transmitted by different *Hyalomma* species, the full vector range for *B. occultans* has not been elucidated. The reports of clinically inapparent or mild infection are certainly consistent for all three parasites (Oosthuizen et al., 2008).

Therefore, according to Romanian literature towards babesiosis in livestock, *Babesia bigemina*, *B. major, B. divergens* have been reported as parasitic species in cattle (Cernaianu, 1957; Feider, 1965; Suteu, 1970), thus further researches are required to clarify the presence of this new *Babesia* species and the epidemiological role of *Hyalomma* ticks.

Regarding to the positive samples for *Rickettsia*, the NCBI BLAST analysis revealed 99% homology with *Rickettsia raoultii*. This species was first identified in *Rhipicephalus pumilio* and *Dermacentor nutalli* ticks collected in southern regions of the former Soviet Union (Rydkina et al., 1999), and was subsequently detected in *D. reticulatus*, *D. niveus,* and *D. silvarum* ticks from various regions of he former Soviet Union (Shpynov et al. 2001), *D. marginatus* ticks from France (Sanogo et al., 2003) and Spain (Merino et al., 2005), *D. reticulatus* ticks from Germany (Dautel et al., 2006) and Netherlands (Nijhof et al., 2007). This species *Rickettsia raoultii* has been suspected to be a second etiologic agent of TIBOLA (tick-borne lymphadenitis) syndrome, besides *R. slovaca*, which is the main etiological agent of this syndrome (Ibarra et al., 2006).

Consequently, whether the pathogenicity of this *Rickettsia* is confirmed or not, the risk of developing TIBOLA in persons bitten by a *Demacentor marginatus* seems to be high, according to literature data (Toledo et al., 2009).

The results discussed in this paper represent the first study on molecular screening of ticks for tick-borne pathogens performed in Romania. These emphasize differences in the role that different tick

species play in the pathogen's life cycle. Moreover, the results are very important for the epidemiological studies of tick-borne pathogens and tick-borne diseases in Romania, but as well as for evaluating the associated risks with pathogen transmission to humans and animals.

## 3. CONCLUSIONS

3.1. The prevalence of tick-borne pathogens (*Babesia, Theileria, Rickettsia* species) was determined in questing and feeding adult ticks for first time in Romania.

3.2. A total of 165 ticks (40 questing ticks and 125 feeding ticks - collected from naturally infested cattle, sheep or goats) from the South of Romania were analyzed. Tick species included *Ixodes ricinus, Dermacentor marginatus*, *Rhipicephalus bursa* and *Hyalomma plumbeum plumbeum*.

3.2. *Babesia equi* was detected in 2.5% of questing *Hyalomma plumbeum plumbeum*.

3.3. Of the feeding adult ticks analyzed, 10.48% were found infected with different pathogens: *Babesia* spp. was detected in 3.99% of *Hyalomma plumebum plumbeum*, *Theileria* spp. was detected in 6.42% of *Hyalomma plumebum plumbeum* and in 33.33% of *Ixodes ricinus; Rickettsia* spp. was detected in 16.6% of *Dermacentor marginatus.*

3.4. The results are very important for the epidemiological studies of tick-borne pathogens and tick-borne diseases in Romania, but as well as for evaluating the associated risks with pathogen transmission to humans and animals.

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# VIRULENCE GENOTYPE OF *PASTEURELLA MULTOCIDA* STRAINS ISOLATED FROM ATROPHIC RHINITIS IN SWINE

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**Keywords:** *Pasteurella multocida*, PCR, swine, atrophic rhinitis

**SUMMARY**

*Pasteurella multocida* is the causative agent of numerous relevant diseases worldwide like hemorraghic septicemia in cattle and buffaloes, enzootic bronchopneumonia in cattle and sheep, fowl cholera, snuffles in rabbit, atrophic rhinitis in swine.
Human infections with *P. multocida* predominantly occur following cat and dog inflicted injuries resulting in cellulitis and lymphangitis, sometimes complicated by abcess formation and septic arthritis.
In this study *P. multocida* isolates were analyzed to discover the presence of the coding gene for the toxin (toxA) using protocol based on PCR assay, previously described by Lichtensteiger and col. (1996), which was later improved.
In this communication we explored the feasibility of PCR for accurate rapid detection of *P. multolcida* from swabs.
We show that for our reaction conditions, PCR is specific and sensitive for toxigenic *P. multocida.* Sensitivity appear increased over reactions for a direct specimen assay. We show that toxigenic *P. multocida* is recovered efficiently from inoculated swabs without inhibiting the PCR assay.
The results show that PCR detection directly from swab specimen should significantly enhance the identification of pigs infected with toxigenic *P. multocida*.

*Pasteurella multocida* is a causative agent of numerous economically relevant diseases worldwide, like enzootic bronchopneumonia in cattle and sheep, hemorrhagic septicemia in cattle and buffaloes, atrophic rhinitis in swine, snuffles in rabbits, and fowl cholera.

Human infections with *P. multocida* predominantly occur following cat and dog inflicted injures resulting in cellulitis and lymphangitis, sometimes complicated by abcess formation and septic arthritis.

*Pasteurella multocida* is part of the commensal flora in the upper respiratory tract of pigs. The bacterium induces pneumonia in grower and finisher pigs, usually as a secondary pathogen invading lungs

injured by other bacteria or viruses. A subset of *Pasteurella multocida* isolates are critical agents in an upper respiratory disease, progressive atrophic rhinitis. This isolates synthesize a 145 Kda toxin encoded by a chromosomal toxA gene. The tox A protein is an essential virulence factor for progressive atrophic rhinitis. The toxin induces turbinate athrophy and poor weight gains in pigs.

Toxigenic *P. multocida* is unintentionally spread to uninfected herds via the addition of asymptomatic infected breeding stock and many aspects of toxigenic *P. multocida* epidemiology and ecology remain unknown, in part, because of the lack of a rapid, sensitive assay to confirm infection. Despite extensive research activities including the genome analysis of one fowl cholera isolate in 2001 leading to the identification of several new potentially virulence associated genes there are a lot of open questions concerning the molecular pathogenic mechanisms of these bacterial species. Problems encountered are the high antigenic variability and the wide host spectrum of *P. multocida* as well as different courses of infection which also imply enormous difficulties in producing vaccines. A more rapid, accurate detection assay is needed for sound decisions regarding diagnosis and treatment to prevent unintentional introduction of infected pigs into clean herds, to support basic studies in ecology and epidemiology of the organisms and to develop more efficacious vaccines. Assays based on PCR are contributing to diagnostic microbiology. Direct specimens analysis using PCR for toxigenic *P. multocida* should be a more rapid and sensitive assay than the free step process of bacterial isolation, biochemical identification and toxigenic testing of isolates.

In this communication we explore the feasibility of PCR for accurate rapid detection of *P. multocida* from swabs.

We conclude that PCR detection directly from swab specimens should significantly enhance the identification of pigs infected with toxigenic *P. multocida.*

The aim of this study was thus to investigate a distribution of so far known virulence associated genes in *P. multocida* strains to find epidemiological associations between.

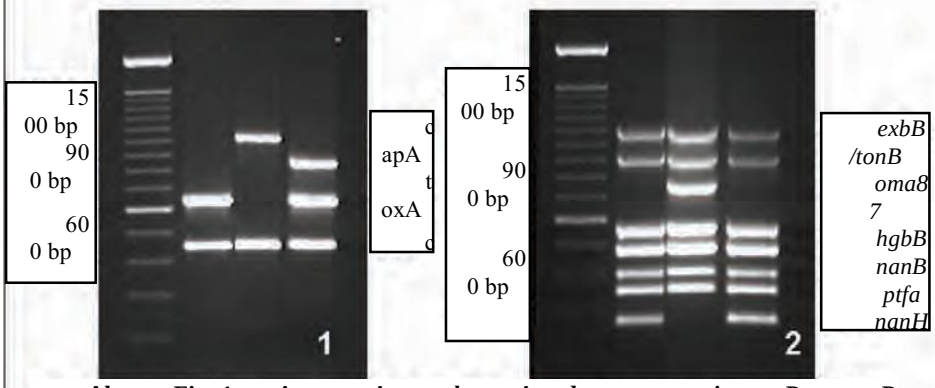
In this study the *P. multocida* isolates were analyzed for the presence codante toxin gene (toxA) using a PCR-based protocol, previously described by Lichtensteiger et al. (1996), which were made a few changes.

## 1. MATERIALS AND METHODS

**Bacterial DNA.** To obtain cells for analysis of P. multocida by PCR was used a 24-hour culture on agar with 5% sheep red blood cells.

Bacterial DNA was obtained by heat lysis (100°C, 5 minutes) of a bacterial suspension in 200 ml of a 10% Chelex-100 (BioRad) in TE buffer (10 mM Tris-HI, 1 mM EDTA, pH 8 ).

**Fig. 2 Identification of *Pasteurella multocida* isolates by Multiplex-PCR assays**




**Abrev.: Fig. 1** ***capA = type A capsule; toxA = dermonecrotoxin; capD = type D capsule; kmt = P. m. specific;*** **Fig. 2** ***exbB/tonB = iron acquisition system; oma87 = outer membrane protein; hgbA/B = hemoglobin binding proteins; nanB/H = neuraminidases; ptfA = type 4 fimbriae; pfhaB = Pasteurella filamentous hemagglutinin***

**PCR for *toxA*.** PCR reactions were performed in a final volume of 50 ml, using a mixture of 0.2 mM of each of the four deoxinucleozidtrifosphatase, 1.5 U Taq polymerase (Roche), 1X reaction buffer, 30 pmol each one primer (toxA1: 5'-CTTAGATGAGCGACAAGG-3 'and toxA2: 5'GAATGCCACACCTCTATAG-3') and 4 ml of bacterial lised. The amplification program consisted of 35 cycles as following: denaturing phase at a temperature of 94°C for 30 seconds, bringing the 55°C for 30 seconds and elongation at 72°C for 1 minute. In addition, we have added an initial denaturing step of DNA matrix for 3 minutes at 95°C and a final amplicons elongation step for 5 minutes at 72°C.

Detection of amplification product (amplicons 846 bp size) was made by electrophoresis in 1.5% agarose gel in 0.5 x TBE, ethidium

bromide staining (10 mg/ml) and photography during exposure to UV (see Figure ). The size of amplicons was assessed by comparison with molecular size marker DNA fragments (100 bp, Promega).

## 2. RESULTS AND DISCUSSIONS

To validate the PCR assay, it must be specific for the toxA gene of *P. multocida*, applicable to all toxigenic *P. multocida* isolates, and sensitive enough to detect just a few organisms. The toxA primes were tested in the PCR essay the toxigenic M33 and non toxigenic M29 *P. multocida isolates*. The expected amplicon was detected in the reaction product of *P. multocida* M33, in agarose gel and stained with ethidium bromide. To determine the accuracy of PCR detection of toxigenic isolates, 25 diagnostic swine isolates of *P. multocida* were screened first, this isolates were characterised by using for other assays to define their toxigenic status. Colony blot hybridization with an internal 22-oligonucleotide toxA probe was used to determine which of this isolates encoded the toxA gene. Biological toxigenic activity of each isolate was tested in a mouse lethality assay. There was concordance of detection of toxA protein in the immunoblot, toxA gene in blot hybridization and mouse lethality of sonicates. 3 of 16 type A and 10 of 16 type D isolates were defined toxigenic by all 3 assays and all other isolates and non-toxigenic by all 3 assays.

Bacterial sonicates were also tested for toxin in an ELISA with the same anti-toxin antibody as used in the immunoblot. ELISA results agreed well with the results 3 assays except that isolate 2 was negative by the ELISA. The results of PCR analysis are in complete agreement with the colony hybridization, colony-immunoblot and mouse lethality results, indicating good specificity of PCR. To be useful and sensitive for research or diagnostic purposes, there needs to be an efficient means to recover *P. multocida* from nasal and tonsillar swabs.

The assay needs to minimize sample handling for efficiency and for avoiding cross-contamination of samples. Essentially, all inoculated *P. multocida* was recovered from the swabs.

## 3. CONCLUSIONS

1. The results indicate that our PCR assay is specific and sensitive. Three assays were run to define true positive (toxigenic) and true negative (non-toxigenic) isolates. Except for two apparent false negative ELISA results, there was complete agreement of the toxigenic status. Of

the *P. multocida* isolates based on colony hybridization of toxA, colony-immunoblot and ELISA for toxA and mouse lethality of sonicate.

2. Regulation of toxin expression is unknown; Our study suggest that under laboratory conditions all toxA positive isolates express toxin.

3. Our colony hybridization and PCR results suggest that this genetic assays are more specific than the colony immunoblot protein assay. In addition our evidence indicates that a PCR assay should be adaptable to direct specimen testing without prior isolation of P. multocida, which was necessary in the other protocols.

4. The results from the 20 isolates indicate that the reactivity is not restricted by variability of tox A gene among isolates or capsule type. In related PCR experiments not shown, using *P. multocida* primers external to toxA, we came to the same conclusions as Lichtensteiger at all, that toxA is in the same location in the chromosome of both capsule type A and D isolates.

5. Our PCR protocol is less labor-intensive and avoids hazardous chemicals used in B. pertussis protocols in which DNA is extracted or the sample digested with proteinase K prior to PCR.

6. Some pig swab specimens contain blood and we have found in pilot studies that one wash does not always effectively lyse the erythrocytes and remove the hemoglobin. Haem of haemoglobin is a potent inhibitor of Taq polymerase.

7. Development of a rapid, valid assay for toxigenic *P. multocida* in nasal and tonsil swab specimens we not only facilitate rapid clinical diagnoses and prompt therapy but will also facilitate epidemiology studies and screening to prevent transmission to clean herds by animal movement.

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# RADIOLOGICAL DIAGNOSIS IN EXPERIMENTAL RABBIT ENDOCARDITIS

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**Keywords:** rabbit, endocarditis, catheter**.**

**SUMMARY**

Heart catheterization in rabbits for experimental purposes can, in time, cause endocarditis, because of the catheter acting like a irritating factor. The orientation of the endocarditis diagnosis can be done through a radiological examination while the confirmation or refutation of the endocarditis type can only be demonstrated by a morphopathological exam. Starting from this consideration, the rabbits used to demonstrate this hypothesis can be used as sentinels to determine the degree of sanitation of medical centers that perform heart surgery.

Radiological diagnosis, along other physical imaging methods of imagistics provide the clinician an element of certainty in the diagnosis. Cardiac catheterization is an invasive method of cardiovascular investigation carried out by introducing probes (catheters) into the heart cavities [4, 5, 7]. The route of introduction may be a peripheral vein for the right heart investigation, or a peripheral artery for left heart investigation. The catheter can obtain accurate and truthful information on the heart, coronary arteries, aorta and any vessel from any part of the body, providing data needed to establish an interventional strategy that is often essential for diagnosis and treatment of cardiovascular disease. Catheter movement within the body is painless and risk of complications during the procedure is very low. Although extremely rare, the following is quoted in the literature: puncture site bleeding, cardiac arrhythmias, valvular or parietal endocarditis, allergic reactions to the contrast substance, infection, obturation of the punctured artery, arterial wall trauma, heart attack cerebral embolism, gas embolism, death [1, 2, 3, 6].

## 1. MATERIAL AND METHOD

Experimental investigations were conducted in the laboratory of Radiology and Surgery Clinic of the Faculty of Veterinary Medicine Cluj-Napoca, in the laboratories of Radiology and Imaging Laboratory

and the Rehabilitation Hospital of Cluj-Napoca and biobase of the University of Medicine and Pharmacy, Targu Mures.

The biological material used in this study was represented by a group of seven rabbits of different ages and sexes, body weight between 2.5 to 3 kg, diagnosed clinically healthy. Each individual was weighed to determine the protocol of anesthesia that could be used and the dose required. Rabbits were subjected to general anesthesia achieved by administration of a intramuscular tranquilizer and a dissociative anesthetic, represented by xylazine solution (5 mg / kg) and ketamine hydrochloride solution (35 mg / kg).

To perform catheterization we used a catheter Vygon 20G type, with a diameter of 0.6 mm, 0.9 mm outer diameter and length of 80 mm.

*Working protocol*. Before the actual intervention, rabbits were restrained in latero-lateral decubitus on the operating table using gauze bandages (Fig. 1), then subjected to X-ray examination to show the normal appearance of the heart and possible heart problems. Individuals with heart disease were excluded from the experimental group. Before the intervention, the right jugular area was prepared by thorough washing with Betadine soap, trimming and shaving, using a clipper and an epilating cream with an astringent and disinfectant effect (Fig. 2). To reduce intra-and postoperative trauma, in addition to general anesthesia, local anesthesia was used through subcutaneous injection of lidocaine 2% solution (Fig. 3). The catheter was inserted in the right common carotid artery through a longitudinal incision of 3-5 cm, it was conducted by the left ventricle through the aortic valve (Fig. 4). The right common carotid artery is placed in the jugular gutter delimited my the neck muscles (cleidomastoidian m., omohyoid m., sternocephalic m.) (fig. 5, fig. 6). Highlighting the common carotid artery requires proper removal of adhering perivascular tissue, but without affecting the vagus nerve or adjacent nerve threads. Inserting the catheter is performed with special care inside the heart, tapping into the heart is signaled by the resistance encountered in the sigmoid valves, and the characteristic pulsation transmitted on the shows that the catheter is placed inside the left ventricle. (Fig. 7 and Fig. 8). From this point, the catheter should be withdrawn about 3-5 mm, so that its tip is positioned on the top of the aortic sigmoid valves.

**Fig. 1. Rabbit restraint on the operating table**

**Fig. 2. Shaving the hair off**

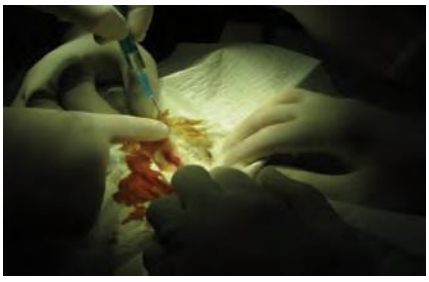

**Fig. 3. Injecting Lidocain 2% solution**

The catheter placed in the heart is fixed with a suture on the proximal end of the common carotid artery. Inserting the catheter and the carotid ligation must be performed quickly in order to minimize blood loss. If there is no bleeding, the skin incision is sutured in separate points in a U-shape, after which the wound needs to be cleaned and Terramicin spray applied (Fig. 9). The fixed catheter is left for 7-14 days, intracardiac. Maintaining the catheter within the heart leads in time to the occurrence of endocarditis.

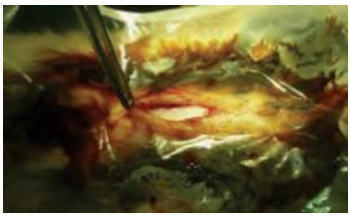

**Fig. 4. Longitudinal skin incision, right side of the inferior cervical region regiunii cervicale inferioare**

**Fig. 5. Evidentiation of the carotid artery**

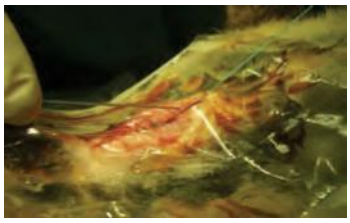

**Fig. 6. Ligation of the carotid artery and catheter introduction**

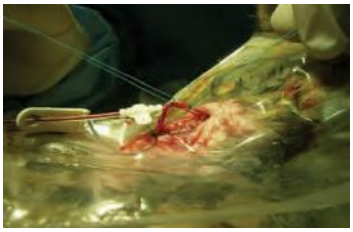

**Fig. 7. Introduction and fixation of the catheter**

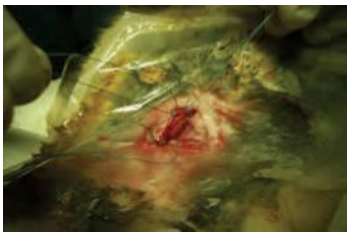

**Fig. 8. Fixation of the catheter**

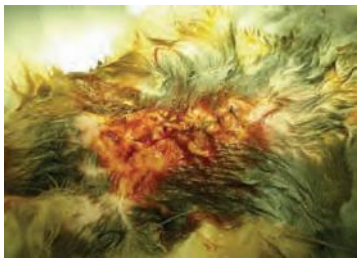

**Fig. 9. Closing up the wound**

## 3. RESULTS AND DISCUSSIONS

The results are interpreted on the basis of preoperative and postoperative radiological examination and macro-and microscopical pathology examination.

*Preoperative radiologic examination.* Following pre operative examination of the animal study group, no cardiac or vascular disease were diagnosed that could alter the study results. For the radiological exam, the rabbit was placed in the latero-lateral decubitus, with the limbs drawn anterior, to provide a more detailed thoracic regions (Fig. 10). The parameters used for chest X-ray examination ranged between 52-55 kV and 20-25 mA, depending on the size of the animal.

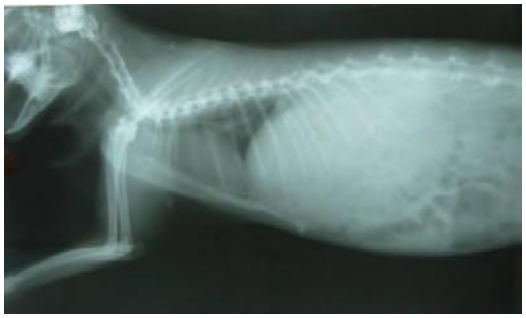

**Fig. 10. Normal aspect of the rabbit heart latero-lateral position, latero-lateral exposure**.

Postoperative radiologic examination. Postoperative radiological examination was performed from the 3rd day after the intracardiac catheter fixation until the 14th day of the experiment. Following radiological examination, we revealed the presence of the catheter (Fig. 11) and base heart opacifying in the projection area of the cardiac valves. In addition, there was an increase in volume of the heart, as evidenced by an increased cardiac shadow. The cardiac shadow on the image occupies three intercostal spaces on the radiological image performed before the intracardiac catheter was inserted, and in the images taken in 7 days of its application, the shadow is projected onto the cardiac thoracic intercostal spaces 4-5 (Fig. 12, Fig. 13, Fig. 14). Cardiac changes that were noticed in the first exposure, and become more clearly outlined after 7 days. In the base of the heart, the left atrium area is separating the left ventricle (Fig. 12) after 3 days from the intracardiac catheter fixation, in the radiographic image opaque areas areas are observed, independent of each other. Right side of the heart (right atrium and right ventricle) being in direct contact with the sternebrae, the radiological image shows that the cardiac axis is oriented almost horizontally cranio-caudal and the area of contact between the heart and sternum is extending more than 3 sternebrae , which shows a dilated heart.

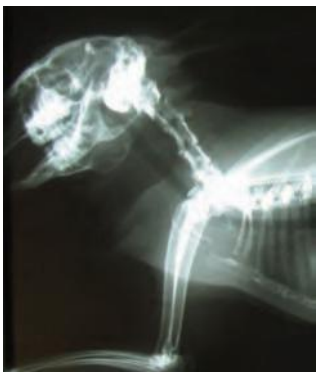

**Fig. 11. radiographic image of the catheter in the carotid artery**

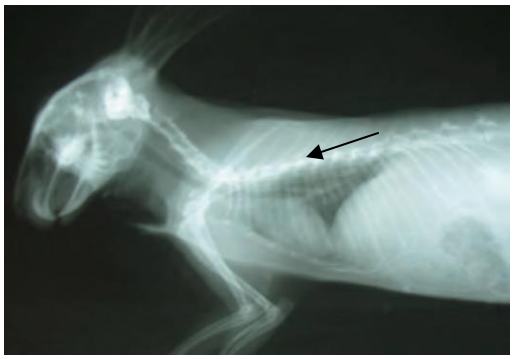

**Fig. 12. Radiographic image of the heart and the catheter in 3 days from fixation, latero-lateral exposure**

Radiographic appearance of heart base shows the appearance of an inflammatory process in the atrio-ventricular valve, due to the presence of the catheter. The catheter acts as an irritating factor, which causes thickening with hyperplasia of the contact structures. Changes can be also seen in the aorta. After seven days after the intracardiac catheter

fixation, on the radiological image one can observe a confluence of the radiopaque areas, resulting in a clear separation between the atria and ventricles (Fig. 13). The left side of the heart is affected more strong, but changes also extend on its right side. The shadow of the heart still changes its shape and the apex of the heart is strong and slightly rounded and ventro-dorsal oriented as a result of bilateral dilation. Radiologically, one observes also a clouding of the right atrium, a sign that at this level an irritating factor has acted, aspect which we connected the cardiac revolution, which led to the displacement of the catheter from the ventricle to the atrium and vice versa.

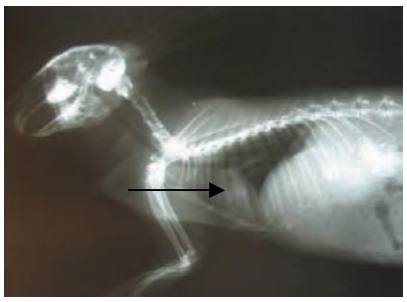

**Fig. 13. . Radiographic image of the heart and the catheter in 7 days from fixation expunere latero-laterală**

From the radiological point of view, after 14 days of catheter fixation, the zone diagnosed opacifying persists, it becomes firm and well defined. Densification and areas of opacifying have been identified in the lungs. The densification can appear due to a possible pulmonary respiratory failure, cardiac failure, or may be due to local vascular parietal reactions.

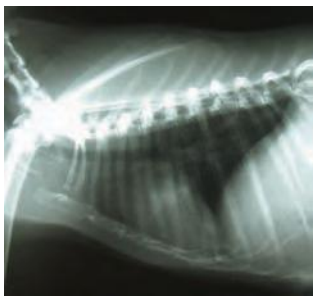

***Fig. 14. Radiography aspect of the heart in 14 days after catheter placement***

**Fig. 15. Catheter evidentiation after herta dissection**

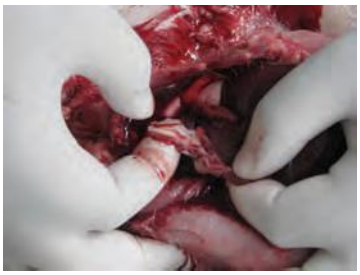

**Fig. 16. Catheter evidentiation in the aorta**

*Pathology exam*. The rabbits in the studied group were sacrificed and the heart and great vessels were examined by macro-and microscopic pathology. Dissection also showed where the stent of the catheter has reached within the heart (Fig. 15, Fig.16).

Macroscopic pathologic examination confirmed the presence of heart endocarditis, endocarditis vegetation development was determined based on the level were the catheter stent arrived.

Dissection of the heart in three cases in the study group revealed vegetant endocarditis localization in the aortic sigmoid valves, lesion severity being determined by the time the catheter stent was maintained at that level. Sigmoid valves presented macroscopic vegetation, they are bold, have a gelatinous appearance and a fine film of fibrin (Fig. 17, Fig. 18).

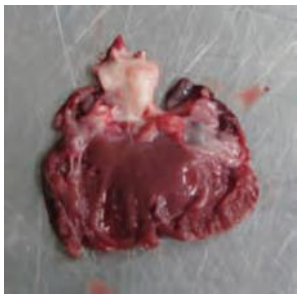

**Fig. 17. Gelatinous aspect of the sygmoid valves**

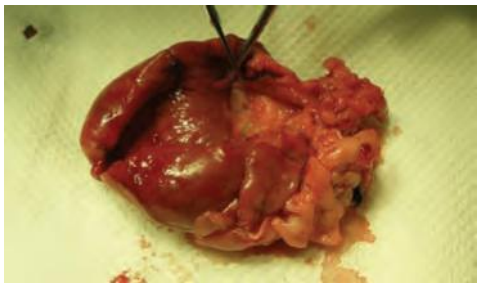

**Fig. 18. Vegetation aspect in the sygmoid valves**

In four of the seven individuals studied, endocarditis lesions were identified in the left ventricular wall, which are also covered by a thin film of fibrin (Fig. 19, Fig. 20).

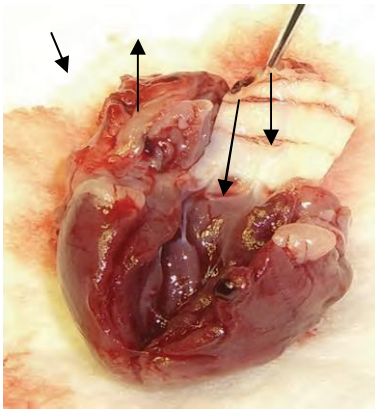

**Fig. 19. The presence of fibrin in the valves and ventricular wall**

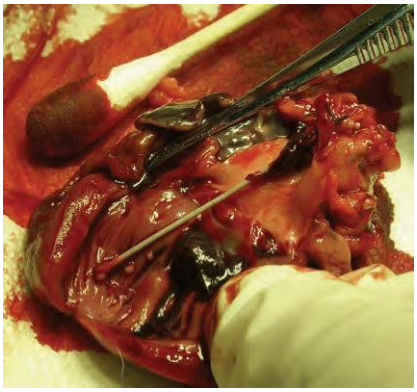

**Fig. 20. Vegetations in the ventricular wall**

Microscopic examination of the lesions reveal the presence of a fibrinous exudate and the proliferation of loose connective tissue (Fig. 21, Fig. 22). Gelatinous appearance of the sigmoid valve is given by the mucoid degeneration of the tissue (Fig. 21, Fig. 22, Fig. 23). In all cases studied in the aorta were highlighted aspects of cartilaginous metaplasia (Fig. 24). The presence of these lesions provides a fertile ground to the grafting of vegetant endocarditis pathogens and transformation caused by the presence of the catheter within the heart bacterial endocarditis.

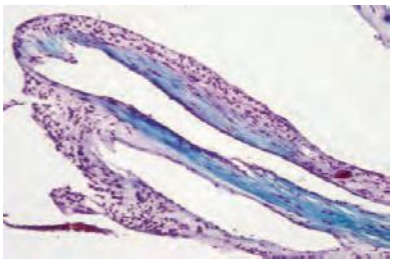

**Fig. 21. Aortic cuspids-loose connective tissue proliferation, collagen degeneration**

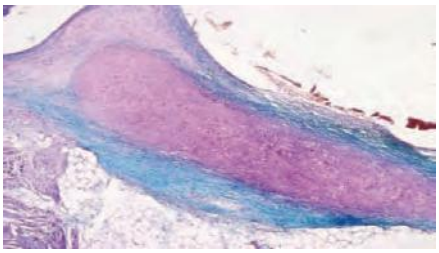

**Fig. 22. Mucoid valvular degeneration (valvular endocariosis) on the aortic cuspids- cartilaginous metaplasia**

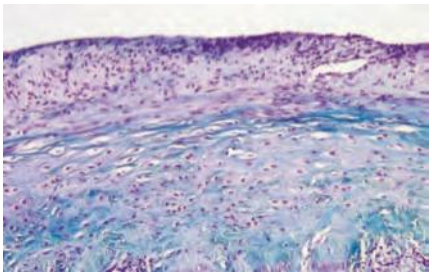

**Fig. 23. Valvular mucoid degeneration (valvular endocardiosis) mixomatous transformations in the aortic cuspids**

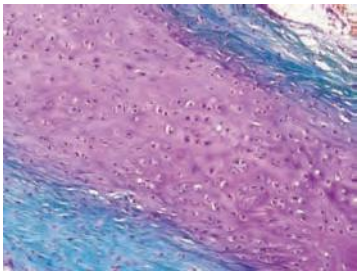

**Fig. 24 Cartilaginous metaplasia in the aorta (vessel origin, aortic sinuses)**

## 3. CONSLUSIONS

Based on the results obtained in the studied group, we can conclude the following:

3.1. intracardiac catheter introduction, for experimental or diagnostic purposes, and its maintenance for a period of 7-14 days can cause valvular or parietal endocarditis, as a local reaction induced by the presence of a catheter that acts as an irritating factor;

3.2. topography of induced changes depends on the level reached by catheter stent;

3.3. the intensity of endocarditis lesions is determined by the period of time the catheter stent was retained intracardiacally;

3.4. radiological evidence of endocarditis lesions is possible from 3 days from the fixation of the catheter;

3.5. in 7 days and 14 days after intracardiac catheter fixation radioopacity lesions diagnosed by radiological examination are evident as a result of illness chronicity;

3.6. radiography can identify lesions in the aortic bulb and the bronchial tree;

3.7. parameters used to obtain radiographic images ranged between 52-55 kV and 20-25 mA, depending on the size of the animal;

3.8. pathological lesions of vegetant endocarditis were identified in the sygmoid aortic valves and there were also parietal lesions;

3.9. catheter presence as an irritating factor determines on a long period of time cartilaginous metaplasia in the aorta.

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# PRELIMINARY DATA REGARDING DETECTION OF ANTIBODIES AGAINST ENZOOTIC BOVINE LEUCOSIS ON MILK AND SERA SAMPLES

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**Key words**: Enzootic bovine leucosis, serum, milk, ELISA, AGID.

## SUMMARY

In Romania, Enzootic bovine leucosis (EBL) it is a disease in interest, notifiable, included into the annually surveillance program of animals diseases.

Bovines serological surveillance is accomplished through enzyme - linked immunosorbent assay (ELISA) and by agar gel immunodifusion test (AGID) on sera samples.

The aim of this study is to evaluate the detection of antibodies against EBL from milk and serum samples harvested from the same animals, in order to establish a new testing methodology for surveillance on milk samples.

The study has been performed on milk and sera samples, harvested from 17 lactating cows. The evaluation of presence of the BLV antibodies could been done based on preliminary comparative results obtained due bytesting of milk and sera samples with seralogical methodes (ELISA. AGID)

In order to asess the sensibility of ELISA and AGID, has been used the poositive OIE reference standard serum, E 05.

The preeliminary results obtaind by testing milk and sera samples have been analised comparativly. For this propose, have been done statistical analysis of the performance parameters: sensibility (Se), specificity (Sp), repeatability (r), intralaboratory reproducibility (R), coefficient of variation (CV%).

Enzootic bovine leucosis (EBL) is a disease of adult cattle, caused by R*etrovirus*, bovine leukemia virus (BLV). Animals can be infected at any age, including the embryonic stage. (4)

Virus is present in peripheral blood lymphocytes as provirus integrated into the infected cells. Also, it can be found in the cellular fraction of various body fluids: milk, colostrum, etc.). The virus integrates as provirus into the DNA of the host cells and remain till the end of the animal's life. (2). BLV infection in cattle is considered to be life long and produces a persistent immune response. Antibodies directed towards virus are found in both milk and blood as early as 3-16 weeks post-infection and maternally derived antibodies may take up to 6-7 months to disappear.

In this study, detection of BLV antibodies in sera and milk samples, was attempted through serological methods with particular attention paid to comparative sensitivity of enzyme-linked immunosorbent assay on milk and sera samples.

The aim of this study was to establish if the enzyme-linked immunosorbent assay used for the detection of antibodies against EBL from milk and sera samples, harvested from the same animals on various lactation periods, is enough sensitive, in order to establish a new testing methodology for EBL surveillance on milk samples.

## 1. MATERIAL AND METHOD

BLV antibodies detection, has been performed by analyzing the results obtained through serological methods. Milk and sera samples were harvested in the same time from 17 dairy cows, from Giurgiu county's householdings, have been tested comparatively. All the samples comprised in this work, derived from animals with known health status, respectively 16 EBL positive animals and 1 negative. The tested animals, aged between 3,5 and 16 years old (16 animals) and in 1 to 4 lactation period and 1 animal 3,6 year old, lied at the end of lactation.

Skimmed milk samples have been tested integral and in decimal dilutions as well. The initially sera samples testing, had been proceeded to the Giurgiu County Sanitary Veterinary and Food Safety Laboratory during annually EBL surveillance program. Therefore, the animal"s health status has been known at the time of milk and blood samples harvesting for further tests performed into the National Reference Laboratory for Enzootic bovine leucosis, within the Institute for Diagnosis and Animal Health (IDAH).

Blood samples have been harvested in vacuumtainers and after sera appearance, proceeded to segregate it from the clot. Milk samples have been harvested into the tubes 45 ml volume and centrifuged before testing (2000 g x 10 min.), in order to separate the fat and remove it. Afterwards, skimmed milk have been transferred in 15 ml volume plastic tubes.

Milk and sera samples have been tested individually through serological methods as agar gel immunodiffusion test (AGID) and enzyme-linked immunosorbent assay (ELISA - blocking) used for the detection of BLV antibodies.

The performance parameters for serological tests (ELISA sensitivity), have been assessed by using the OIE positive standard serum for EBL, calibrated, E 05, considered to be the new official

standard serum of the European Union (EU) for enzootic bovine leucosis and produced by the OIE Reference laboratory in Germany (Friedrich-Loeffler-Institute), in collaboration with OIE Reference laboratories from UK (Veterinary Laboratories Agency) and Poland (National Veterinary Research Institute) (1). Comercially ELISA kits sensitivity have been assessed with E05 OIE standard serum diluted 1:10 in negative serum and 1:250 in negative milk into the Virology Laboratory, IDAH.

The obtained results after milk and sera testings, have been analyzed comparatively, through ELISA method. Statistical analysis have been performed for the following performance parameters: sensitivity (Se), specificity (Sp), repeatability (r), intralaboratory reproducibility (R), variation coefficient (CV%). For the repeatability assessement, samples have been placed on multiwell ELISA microplate onto 3 sequential wells, in 2 different days, using the same equipment, reagents, operator. For the intralaboratory reproducibility, samples have been tested with different operators into 2 wells on the same multiwell ELISA microplate, on 3 different days. The variation coefficient have been assessed based on the ratio ratio between standard deviation and arythmetic media for each tested sample.

## 2. RESULTS AND DISCUSSIONS

Antibodies to BLV readily cross the blood milk barrier and were found in the milk at some similar levels to those in serum. ELISA method, enabled detection of all lactating dairy cows with BLV antibodies, from milk and sera samples.

In table no 1, are shown the results obtained due by ELISA and AGID testing of milk and sera samples, for EBL.

***Table no 1***

**ELISA and AGID comparison of results for milk and sera samples, in EBL:**

| ELISA OD | | | | | AGID |
|---|---|---|---|---|---|
| **Dairy cow ID no** | **Sera samples OD** | **Interpretation of sera samples results** | **Milk samples OD** | **Interpretation of milk samples results** | **results for sera samples** |
| 3545 | 0,083 | + | 0,466 | + | + |
| 3525 | 0,112 | + | 0,211 | + | + |
| 6800 | 0,116 | + | 0,449 | + | + |

| | | | | | |
|---|---|---|---|---|---|
| 7126 | 0,08 | + | 0,107 | + | + |
| 6798 | 0,19 | + | 1,676 | - | + |
| 9548 | 0,11 | + | 0,428 | + | + |
| 7052 | 0,1 | + | 0,132 | + | + |
| 5901 | 0,075 | + | 0,14 | + | + |
| 5068 | 0,126 | + | 0,576 | + | + |
| 5910 | 0,081 | + | 0,265 | + | + |
| 6650 | 0,139 | + | 0,126 | + | + |
| 6631 | 0,098 | + | 0,253 | + | + |
| 6874 | 0,1 | + | 0,176 | + | + |
| 6753 | 0,078 | + | 0,161 | + | + |
| 6855 | 0,076 | + | 0,097 | + | + |
| 6867 | 1,642 | - | 2,381 | - | - |
| 6770 | 0,141 | + | 0,585 | + | + |
| E 05 1/10 | 0,445 | + | | | |
| E 05 | | | 0,75 | + | |
| TOTAL | | 16 | | 15 | 16 |

Legend: + = positive;
- = negative.

Due by ELISA testing, 16 of 17 tested sera samples, registered positive results and 1 sample has had negative result to EBL, according with the expected results.

Between ELISA tested milk samples, 15 have had positive result and 2 samples were negative.

The results obtained after sera samples testing with AGID test, registered 16 positive samples and 1 negative.

Owing to ELISA sensitivity, BLV antibodies could be detected till the kit detection limit, onto milk and sera samples, as shown in table no 1:

- Sensitivity of ELISA kit has been evaluated toward with E05 standard serum, which has had positive result on dilution 1/10 for sera samples, according to OIE standard methods Manual.

- Sensitivity of ELISA kit has been evaluated toward with E05 standard serum, which has had positive result on dilution 1/250 for milk samples, according to OIE standard methods Manual.

***Table no 2***

**ELISA and AGID tests sensitivity comparison, based on the results obtained by sera samples testing for EBL:**

| No of tested animals ELISA + | No of tested animals AGID + | No of tested animals AGID - | Sensitivity AGID x 100 % |
|---|---|---|---|
| | | | ELISA |
| TOTAL 16 | 15 | 1 | 93,75% |

The sensitivity of ELISA was 93,75% compared with AGID test, as shown in table no 2. This analyze has been realize based on the obtained results of sera samples testing through serological methods.

Milk samples after ELISA testing, have had registered values close to the test limit detection but slightly decreased in BLV antibodies compared with sera samples.

The ELISA results concordance between milk and sera samples was of 93,75%.

***Table no 3***

**Average of the ELISA obtained results for BLV antibodies detection on milk (including samples dilutions) and sera samples:**

| Dairy cow ID no | ELISA OD | | | | Lactation period |
|---|---|---|---|---|---|
| | Sera samples OD / Results interpretation | Milk samples OD / Results interpretation | Dilutions of milk samples | | |
| | | | 1/10 | 1/20 | |
| 6867 | 0,126 + | 0,576 + | 1,782 | 1,951 | Second month |
| 3545 | 0,139 + | 0,126 + | 1,005 | 1,419 | First month |
| 6800 | 0,1 + | 0,176 + | 1,119 | 1,477 | First month |
| 6798 | 0,076 + | 0,097 + | 0,658 | 1,138 | Fourth month |
| 7052 | 0,141 + | 0,585 + | 1,717 | 1,89 | Last month |

The obtained results due by ELISA testing, have shown a significant correlation between the titre of BLV antibodies within the milk and sera samples for lactating cows (table no. 3).

The performance parameters ranged between the expected values.

## 3. CONCLUSIONS

3.1. The obtained results due by ELISA testing on milk and sera samples harvested from the same animal, were in 93,75% concordance.

3.2. Sensitivity of AGID test compared with ELISA, were 93,75% on sera samples.

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# STUDIES REGARDING ANIMAL ANESTHESIC HIPOTERMIA

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**Key words**: anesthesia, hypothermia, dogs, cats.

**SUMMARY**

It is known that general anesthesia suppress termoregulating centers, decreases metabolism and dilates the peripheral vessels, that the patient becomes almost poikiloterm. If the operating room is cold, body temperature can drop to 32-34 C and affect the physiological constants, leading the body to unpredictable developments, difficult to control.
The mechanisms by which anesthesia have hypothermic effects are multiple and require body temperature monitoring throughout the surgical procedure and anesthesia recovery. It prevents heat loss by removing the casual factors by removing the casual factors, heat loss compensation and by stimulating heat production by appropriate means.
Based on animal clinical observations(dogs and cats) effectual paper proposes several protocols and procedures easy to do in terms of minimal equipment.

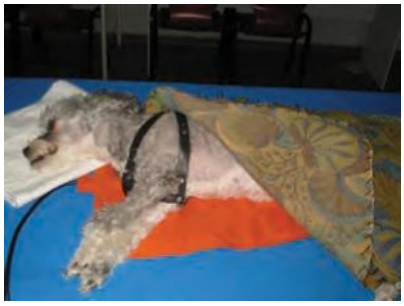
## 1. MATERIALS AND METHODS

Observations have been made over the evolution of body temperature in corelation with the duration and the degree of general anesthesia (NLA) in which the neuroleptical component is assured by acepromazine, know for its disruptive effect on thermoregulating mechanisms.

The animals, cats and dogs, were monitorized before, during and after the implementation of surgerical procedures observing the changes in body temperature until the physiological parameters were fully re-established.

The temperature in the operating room was kept between 22 to 24 $^{o}$ C and the degree of anesthesia was maintained accordance with the length and magnitude of the operation.

Constant monitoring of vital functions was realized through recording of basic parameters: respiration, pulse, temperature in corelation with the degree of anesthesia, for each animal.



In the instances where anesthesia is kept at a deep enough grade to enable surgical procedures to be performed and until the complete recovery of the patient to a vigilant state, different procedures were applied which had three objectives in mind:

-the reduction of heat loss

-the re-heating of the body

-stimulation of the body's heat production

## 2. RESULTS AND DISCUSSIONS

| 0. | Species / Age / Diagnosis / Body Weight | Anesthetic and surgical procedure | Body temperature $C^{O}$/minute | | | | | | | COMMENTS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 15 | 30 | 60 | 120 | 180 | 360 | Min. 0 = începutul procedurilor anestezice |
| | **01** | **02** | **3** | **0 4** | **5** | **6** | **7** | **8** | **9** | **10** |
| | Dog, Cocker, 14 years old. Mesenchymal tumor in the mouth, 15 kg | NLA – Sedam + Vetased ; Abscission | 39 | 39,2 | 38,5 | 38,0 | 38,2 | 38,2 | 38,6 | Thermal rebalancing procedures were applied in minute 60, for four hours |
| | Dog, Romanian shepherd metis, 6 months old, Bilateral fractures of the tibia, 10 kg | NLA – Sedam + Vetased ; Osteosintesis | 39,5 | 39,6 | 37,5 | 38,0 | 38,3 | 38,5 | 38,4 | There was a decrease in temperature to two degrees C after 30 min. Back in 15 min. By injection means. |
| | Dog, metis, 10 years old, tumor ( mastocitom ), humero-radio-ulnar reg, 25 kg | NLA – Sedam + Ketaminol ; Abscission | 38,8 | 38,2 | 37,4 | 37,3 | 37,5 | 37,5 | 38,2 | Resuscitation from min. 15, blood loss amid a hepetic shortfall. |
| | Dog, Boxer, 12 months old, Open right femur fracture. 30 kg | NLA – Sedam + Ketaminol ; asepsis and osteosintesis | 37,8 | 37,8 | 38,2 | 38,2 | 38,5 | 38,8 | 38,7 | Complex thermal rebalancing while anesthesia. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dog, Doberman, 9 years old, Mouth papilomatosis, 28 kg | NLA – Sedam + Vetased ; Thermal cautery | 38,9 | 38,7 | 38,4 | 38,2 | 38,4 | 38,6 | 38,6 | Heat loss was limited without additional heating |
| | Dog, German shepherd german, 7 years old, Fractură dentară complicată cu fistulă facială, 37 kg | NLA – Narcoxil 2 + Ketaminol Trephinning, bandaging, extraction, drainage | 39,5 | 39,5 | 38,2 | 38,2 | 38,7 | 38,8 | 38,8 | Sepsis, risk of sepsis, rapid recovery and balancing |
| | Dog, Metis, 10 years old, Intestinal obstruction, megacolon, 19 kg | NLA – Sedam + Vetased ; Enterectomy | 37,5 | 37,2 | 37,5 | 38,0 | 38,2 | 38,1 | 38,4 | Laborious operation, with greater injury, balancing heat with all available means. |
| | Dog, Doberman, 7 years old, Dilataţie şi torsiune gastrică, 35 kg | NLA – Domitor + Butomidor ; Antisedan ; Gastropexis, Splenectomy | 40,4 | 39,4 | 39,2 | 38,2 | 38,2 | 38,3 | 38,2 | Bad status. difficulty rebalancing, risk of kidney failure, infusion (4l/24 h) |
| | Dog, Metis, 4 years old, Intrathoracic oesophageal obstruction, 18 kg | NLA – Domitor + Butomidor ; Antisedan ; Gastrotomy | 39,6 | 39,8 | 38,2 | 38,0 | 38,5 | 38,5 | 38,6 | Sudden decompensation, immediate action, slow recovery in body temperature |
| **0** | Dog, Great dane, 12 years old, Pelvis and femoral neck fracture, 55 kg | NLA – Narcoxil 2 + Ketaminol Pelvis and femoral neck osteosynthesis | 37,4 | 37,8 | 38,2 | 38,3 | 38,2 | 38,4 | 38,6 | Stabilized internal bleeding. Volume and thermal rebalancing. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | Cat, Burmese, 11 years old, infected mammary tumor, 4 kg | NLA – Sedam + Ketaminol Mammary gland tumor ablation | 39,6 | 39,5 | 38,2 | 38,2 | 38,1 | 38,2 | 38,7 | External heating, fluid balance |
| **2** | Cat, Siamese, 6 years, open femur fracture, bilateral, 5 kg | NLA – Narcoxil 2 + Ketaminol asepsis and osteosintesis | 37,6 | 37,2 | 37,6 | 38,2 | 38,4 | 38,4 | 38,6 | Stare precomatoasă, recuperare dificilă, se aplică toate mijloacele disponibile |
| **3** | Pisică, Europeană, 8 months, Multiple wounds, bitten and infected, 4 kg | NLA – Domitor + Butomidor; Antisedan Asepsis and sutures | 37,4 | 37,2 | 38,2 | 38,2 | 38,4 | 38,6 | 38,5 | Severe posthemoragic anemia, fluid and thermal rapid rebalancing |
| **4** | Cat, Metis, 13 years old, Humerus osteosarcoma with subsequent fracture, 5 kg. | NLA – Domitor + Butomidor; Antisedan Total amputation of the front leg. | 39,8 | 40,0 | 38,0 | 38,2 | 38,7 | 38,6 | 38,7 | Anesthetic decompensation, rapid recovery, and thermal fluid rebalancing |

Methods towards thermal reestablishment were applied in concordance with the gravity of the hypothermia and was continued until the re-stabilization and maintenance of normal values.

- Light Hypothermia – passive re-heating methods were applied, the covering of the body with warm fabrics, the vital systems were monitored, and the reduction in body heat loss. Massage of the extremities for the aid of peripheral circulation was also performed.
- Moderate Hypothermia – external active re-heating methods were applied, such as electric pillows and radiant heat. Heat was applied in the trunk region for the reheating of the middle of the body without producing peripheral dilatation of the limbs. A protective layer between the source of heat and the patient's body was assured.

- Severe Hypothermia – body re-heating procedures were applied, including warm-water enemas, and i.v. administration of electrolytic solutions at a temperature of $37^{o}$ - $38^{o}$ C, especially of calcium gluconate. The placement of warm objects (bottles or bags of warm water, electric pillows, etc) and the reheating of the airways with warm air were proven to be simple and efficient methods of alleviating hypothermia.

Throuout the whole thermal resuscitation efforts, the respiratory and cardiac rates were monitored, following the blood pressure values through the degree of tissular perfusion knowing that the reduction of cellular metabolism can lead to a protective effect for animals with severe hypothermia.

## 3. CONCLUSION

3.1 Normal physiological functions such as body temperature, respiration and cardiovascular function need to be monitored and sustained throughout the duration of the anesthesia, until the complete recovery to normal physiological parameters.

3.2 Anesthetic hypothermia is permanent, with very large individual variations and can influence the evolution of the surgery, during and afterwards.

3.3 Monitoring of body temperature alongside cardio-pulmonary functions, starting with pre-anesthesia and following throughout the duration of the surgery assures an efficient way of controlling the evolution of the surgical procedure.

3.4 Anesthetic combinations based on the major tranquilizer group have proven to have a higher hypothermic potential due to the peripheral vasodilatation that facilitates the loss of temperature.

**3.5** The methods of re-establishing on anesthetized patients are chosen differentially according to the hypothermic mechanisms of the anesthetics and the degree of destabilization**.**

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# RETROSPECTIVE STUDY ON URINARY TRACT DISEASES IN CATS IN A VETERINARY CLINIC

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**Key words:** urinary tract, cat, prevalence

**SUMMARY**

In the period comprised between November 2009 and April 2010, in the Agervet Clinic-Târgovişte were diagnosed 57 cats with diseases of the urinary apparatus, out of which there were 31 males and 26 females with ages ranging from 1 to 13 years. Our clinic determined different diagnoses among which we can mention: diseases of the upper urinary tract, including kidneys and ureters - 31.58% (18/57), but especially and more often diseases of the lower urinary tract, including the urinary bladder and the urethra - 68.42% (39/57). The symptomatology of the urinary system included nephrites - 26.31% (15/57), cystitis - 31.58% (18/57), urolithiasis - 42.11% (24/57).

The affections of the urinary system may carry different aspects and can be classified within the affections of the upper urinary tract and affections of the lower urinary tract. The evaluation of the urinary tract meant correlating data obtained through general methods of examination with paraclinic exams. The imagistic examination represents a frequent method used in veterinary practice for investigation of the urinary system and some pathologic processes. The radiology images allow the evaluation of the relationship between the urinary tract affections and clinical signs (Temizsoylu et al., 2006). The normal radiology image of the kidneys is influenced by the age of the animal and the general condition of the body. Usually, kidneys can be visualised on the radiology image but the intestines can come in front and cover them partially or totally. The ureters can't be visualised in normal conditions due to their small dimension. The urinary bladder can be individualised in the vicinity of the pelvis edge. Utrasonographic exam can complete the results obtained through radiology and offer important data about the location, shape, size and ecostructure of the urinary apparatus segments (Codreanu, Diaconescu, 2003). This paper presents an evaluation of the prevalence of the urinary apparatus in cats inside a private clinic.

## 1. MATERIAL AND METHODS

In the period comprised between November 2009 and April 2010, inside Agervet Clinic-Târgovişte, 57 cats were diagnosed with diseases of the urinary apparatus, of which 31 were males and 26 females with ages ranging from 1 to 13 years. The animals were examined clinically through general (inspection, palpation) and complementary methods (radiographic and ultrasonographic exam). History was obtained from each animal owner.

## 2. RESULTS AND DISCUSSIONS

After the clinical examination, it was found that the urinary tract diseases were present in different forms and symptoms. Our clinic determined different diagnoses among which we can mention: diseases of the upper urinary tract, including kidneys and ureters 31.58% (18/57), but especially and more often diseases of the lower urinary tract, including the urinary bladder and the urethra - 68.42% (39/57). The symptomatology of the urinary system included: nephrites - 26.31% (15/57), cystitis - 31.58% (18/57), urolithiasis - 42.11% (24/57) (Fig.1).

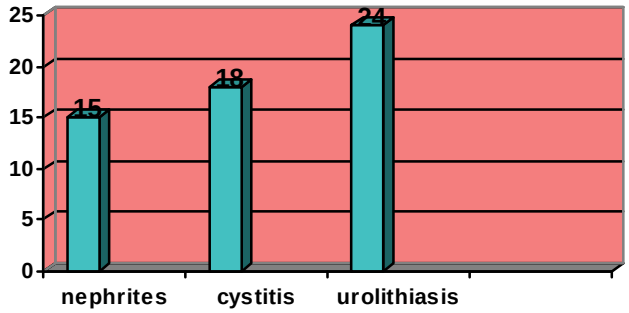


**Fig. 1 - The urinary tract diseases distribution**

In this study it has been noticed that the lower segment of the urinary apparatus is more affected than the upper segment, and that males are more affected than females, 54.39 % to 45.61%. Eugênio et al. (2009), examined a number of 72 male cats with FLUTD (Feline Lower Urinary Tract Disease) and noticed that the highest prevalence belonged to the nuttered ones.

According to our results within this study, the main spotted affection was urolitiasis, easily traceable through imagistic methods. The presence of a high number of urolitiasis cases can be attributed, especially, to administering inadequate diets. Urolitiasis occurs both in females and

males, but is manifested clinically especially in males, due to the anatomic characteristics of the genito-urinary apparatus (Osborne et al., 2000).

Aside our results, Kirk et al. (2001) noticed that 6.6 % of the examined cats showed affections of the urinary apparatus, and the main diagnoses affection was cistitis. In the present study, cystitis can be considered to be closely related to the high number of urolitiasis cases. Cystites generally represent inflamations of the urinary bladder with different ethiologies. Previous studies have suggested that bacterian cystites are rarely encountered in cats, idiopatic ones being more frequent (Sparkes, 2006). Dysuria, stranguria, pollakiuria, hematuria and urinating in other places than the sandbox (inappropriate urination, or periuria) are signs that lead to diseases of the urinary apparatus (Chew et al., 1999). Those signs aren't specific to a disease and can be found in cats with kidney stones, infections of the urinary tract or tumors (Westropp, 2008). Urolites can have different aspects, from sand to large stones, and their presence in the urinary tract act mechanically causing irritation of the urinary epithelium causing varied pathology within the urinary apparatus.

## 3.CONCLUSION

In conclusion, the obtained result in the study shown here have revealed that the urinary apparatus pathology in cats was dominated by diseases of the lower trac, the main affection being urolitiasis.

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# RESEARCHES CONCERNING THE FISH WELFARE ASSESSEMENT BASED ON SERUM BIOCHEMICAL PROFILE IN A CARP FISHERY FROM DAMBOVITA COUNTY

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**Key words:** carp, welfare, biochemical profile

**SUMMARY**

Among the objective indicators of fish welfare, the serum biochemical profile shows most clearly and quickly any environmental and physiological changes which could affect the individuals. The aim of the present study was to assess the welfare level in an extensive carp fishery from Comisani area based on serum biochemical profile. From the farm fishpond there were harvested 3 individuals and after stunning, there was collected blood by caudal vein puncture: 1-2 ml/fish. The samples were transported to laboratory, where there were established by using Vettest 8008 the following serum parameters: blood urea nitrogen (BUN), phosphatemia (PHOS), creatinine (CREA), uric acid (URIC), calcium (CA), magnesium (Mg), total proteins (TP), albumine (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), cholesterol (CHOL), triglycerides (TRIG), glucose (GLU), lactate dehydrogenase (LDH) and alkaline phosphatase (ALKP). The analyze shows changes for some parameters: CA, MG, TP and ALB (in first captured carp), uric acid (in third captured carp), LDH – caused most likely by dehydration and intense muscular activity during fish angling and restraining. In addition, there were registered overvalues for CHOL, ALT and AST (pronounced in first captured fish), GLU – which suggest stress and hepatopancreatic lesions with impact on fish welfare.

Defined by Broom as "the psychological and physiological state as regards its attempts to cope with its environment" [1], animal welfare is lately a topic of major concern both for scientific research and general public. Assuring a good welfare level for animals is not just a way to improve the production, but a moral duty of human regarding animals.

Fish welfare was for a long time controversial (the presence of negative or subjective states in fish issue being argued), but recent researches demonstrate that fish nervous system is similar to mammals', having transmission role for nociception from different body regions to the brain and being implied in stress response [2].

As there is no modern aquaculture without assuring fish good welfare, many scientific projects treating this problem worldwide: Benefish, Wellfish, Fastfish etc.

Similar to other animals of economic interest, fish welfare assessment could be conducted mainly based on ethological indicators

and management system and practices (water quality and stocking density). Although the less studied among the objective fish welfare indicators, serum biochemical profile shows most clearly and quickly any environmental and physiological changes which could affect the individuals. The aim of the present study was to assess the welfare level in an extensive carp fishery from Comisani area based on serum biochemical profile.

## 1. MATERIALS AND METHODS

There was assessed the welfare level in a private fishery from Comisani – Dambovita, from a carp rearing fishpond, 20 m length and 12 m width, with an average depth of 1,25 m (0,5 m at the border and 2 m in the center).

By angling, there were collected 3 individuals belonging to common carp species (Cyprinus carpio), of 250 g average body weight. Water temperature in harvesting time was 18-20 ºC (normal range for carp rearing).

After stunning, there was collected blood by caudal vein puncture: 1-2 ml/fish. The samples were transported to laboratory, where there were established by using Vettest 8008 the following serum parameters: blood urea nitrogen (BUN), phosphatemia (PHOS), creatinine (CREA), uric acid (URIC), calcium (CA), magnesium (Mg), total proteins (TP), albumine (ALB), aspartate aminotransferase (AST), alanine transaminase (ALT), cholesterol (CHOL), triglycerides (TRIG), glucose (GLU), lactate dehydrogenase (LDH) and alkaline phosphatase (ALKP). All parameters were established from undiluted serum, except LDH which was analyzed from serum diluted 1:2.

The obtained results were compared with the data in the literature [3, 4], except for triglycerides (TRIG), where couldn't be found reference values.

## 2. RESULTS AND DISCUSSIONS

The values obtained following the analysis of biochemical parameters are shown in Table 1.

***Table 1***

**Values of biochemical parameters in carp from Comisani fishpond**

| Assessed parameters | Obtained values: carp 1 | Obtained values: carp 2 | Obtained values: carp 3 | Reference values |
|---|---|---|---|---|
| Blood urea nitrogen (BUN) | 3 mg/dl | 2 mg/dl | 3 mg/dl | 1,9-3,6 mg/dl |
| Creatinine (CREA) | 0,1 mg/dl | 0 mg/dl | 0,1 mg/dl | 0,07-0,09 mg/dl |
| Uric acid (URIC) | 1,6 mg/dl | 1,2 mg/dl | 3,6 mg/dl | 1,3-2,5 mg/dl |
| Calcium (CA) | 11 mg/dl | 10,1 mg/dl | 12,4 mg/dl | 8-9 mg/dl |
| Magnesium (MG) | 5,2 mg/dl | 3,62 mg/dl | 3,62 mg/dl | 2,6-3,4 mg/dl |
| Total protein (TP) | 4,3 g/dl | 2,8 g/dl | 3,6 g/dl | 2,5-3,5 g/dl |
| Albumin (ALB) | 1,4 g/dl | 0,5 g/dl | 0,9 g/dl | 0,76-0,85 g/dl |
| Alanine aminotransferase (ALT) | 13 U/l | 9,8 U/l | 9,9 U/l | 8,9-9,9 U/l |
| Aspartate aminotransferase (AST) | 347 U/l | 115 U/l | 127 U/l | 121-124 U/l |
| Triglycerides (TRIG) | 164 mg/dl | 170 mg/dl | 130 mg/dl | N/A |
| Glucose (GLU) | 174 mg/dl | 178 mg/dl | 158 mg/dl | 30-47 (96) mg/dl |
| Lactate dehydrogenase (LDH) | 2565 U/l | 2499 U/l | 4853 U/l | 860-1200 U/l |
| Alkaline phosphatase (ALKP) | 72 U/l | 28 U/l | 38 U/l | 14-24 U/l |
| Cholesterol (CHOL) | 200 mg/dl | 99 mg/dl | 90 mg/dl | 127-184 mg/dl |

Analyzing the data in the table, it can be noticed that blood urea nitrogen, creatinine and uric acid are in normal range, so the excretory function (renal and gills excretion) is normal. However, in the third collected carp was registered an exceeding of uric acid reference value with 44% (3,6 mg/dl), related most likely with dehydration and muscular lesions caused by angling.

Regarding serum ionogram, calcium concentrations (10,1 to 12,4 mg/dl) were with 12,2 – 37,7% higher than reference value in all carps while magnesium registered a significant increase only in first individual (with 52,94%).

For total protein, albumin, alanine aminotransferase and aspartate aminotransferase there were noticed increasing of normal range only in

the first collected carp, with 22,8%, 64,7%, 31,3%, respectively 179,8%. In conclusions, the welfare level in this individual is poor than in the others collected, the transaminase high activity pointing to severe hepatopancreatic lesions.

Glucose, lactate dehydrogenase and alkaline phosphatase registered overvalues in all collected carps: with 64,58-85,4%; 108,24-304,4% and 16,6-200%. The pronounced change of LDH level shows a high level of stress associated with capture and restraining.

Cholesterol level recorded an insignificant increase in the first collected carp (with 8,69%), while in the other the level is lower, most likely as results of malnutrition.

If the most changes of serum biochemical parameters are caused by fish capture and restraining, in the first individual the increasing is pronounced and is certainly related with welfare issues.

## 3. CONCLUSIONS

3.1. The biochemical serum profile shows changes for some parameters: CA, MG, TP and ALB (in first captured carp), uric acid (in third captured carp), LDH – caused most likely by dehydration and intense muscular activity during fish angling and restraining.

3.2. However, the overvalues registered for CHOL, ALT, AST (pronounced in first captured fish) and GLU suggest stress and hepatopancreatic lesions with impact on fish welfare.

*Acknowledgments.* This study has been financed and is a part of ID_285 grant CNCSIS UEFISCSU, contract Idei 290/2007: Researches concerning the welfare of fish in farm fish, in transportation and slaughtering units.

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# WATER QUALITY AS AN INDICATOR FOR FISH WELFARE ASSESSMENT IN A PRIVATE POND FROM DAMBOVITA AREA

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**Key words:** fish, water, indicators, welfare, carp

**SUMMARY**

Fish good welfare reflects their rearing so they could be able to maintain homeostasis, to be protected by stressors, and to allow them a normal somatic development.
From a fish pond in Comisani-Dambovita there were collected water samples in 3 points (water admission point, center and draining) from which were established the physical chemical parameters (pH, $O_2$, $NH_4^+$, $NO_3^-$, $NO_2^-$, P, Fe, chlorine, $SO_4^+$, Cu and detergents).
For analyzing the samples, there was used NOVA60 photocolorimeter device and the results interpretation was made according to the reference values for carp.
Following the researches there can be noticed that the water from Comisani fish pond is proper for carp rearing and reflects good fish welfare.

Animal welfare has represented the subject of many argues since the beginning of their use as farm or favorite animals [1].

Until recently, fish, both from fisheries and captured from their natural habitats, were not took into account by these argues, considering that they don't feel pain as a negative emotion.

Lately, various researchers found out that the central nervous system (CNS) of fish is similar to the CNS of mammals, playing a major role in transmitting pain [2, 3].

In the modern aquaculture fish good welfare means to ensure rearing conditions so they can maintain homeostasis, to have a normal somatic development and to be free of stressors [7].

Of the many factors that influence fish welfare, their living environment has a main role.

## 1. MATERIALS AND METHODS

In a private fish pond from Comisani area - Dambovita County there were conducted studies on water quality, based on physical and chemical

parameters. There were collected water samples from 3 points of the pond: admission, center and draining.

From the above samples, there were analyzed: pH, $O_2$, ammonia ($NH_4^+$), nitrates ($NO_3^-$), nitrites ($NO_2^-$), total phosphorus (P), iron (Fe), residual chlorine, sulfates ($SO_4^{2-}$), copper (Cu) and detergents.

Water samples were collected by using PSB4 harvesting device. The assessment was made by using NOVA60 photocolorimeter and results interpretation was made according to the reference values suggested by Schlotfeldt H. J., 1995.

## 2. RESULTS AND DISCUSSIONS

The results of water quality parameters analysis from the carp pond in Comisani - Dambovita are shown in Table no. 1.

***Table 1***

**The values of water quality parameters – Comisani fish pond**

| Sampling point | No. of samples | Assessed parameters | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH | $O_2$ mg/l | $NH_4$ mg/l | $NO_2$ mg/l | $NO_3$ mg/l | P mg/l | Iron mg/l | Free chlorine mg/l | $SO_4$ mg/l | Copper mg/l | Cationic detergents mg/l |
| Water admission | 10 | 7.9 | 15.3 | < 0.02 | 0.027 | 1.0 | 0.8 | 0.22 | 0.26 | 77.0 | 0.13 | 0.01 |
| Center of the fishpond | 10 | 7.9 | 21.0 | 0.01 | 0.027 | 2.0 | 0.8 | 0.19 | 0.33 | 72.0 | 0.14 | 0.02 |
| Draining | 10 | 7.9 | 24.0 | 0.01 | 0.030 | 1.0 | 1.2 | 0.1 | 0.32 | 71.0 | 0.16 | 0.02 |
| Admitted Limits (Schlotfeldt H. J. 1995) | | 5.5 – 8.0 | Min 4 | Max 1.0 | Max 0.02 | 5.0 | 0.6 – 1 | Max 2 | 0.01 – 0.03 | 80 I 150 II mg/dl | Max 0.3 | 0.05 mg/l |

Analyzing the data in the table it can be noticed that the water pH in all 3 collecting points recorded values close to the maximum admitted limits for carp.

Water reaction is important as it is influencing physical chemical and biological processes as well as the toxicity level of metals and nitrites in the water.

Dissolved oxygen in all samples has values in normal range (over 4 mg/l - the minimum admitted limit), proper for the carp rearing.

Dissolved oxygen represents the water chemical element on which fish life depends. It helps to the mineralization of the organic substances in the water and, along with the pH; it is influencing the toxicity level of some water compounds [4, 5, 6].

Of the nutrients category there were assessed ammonia ($NH_4^+$), nitrates ($NO_3^-$), nitrites ($NO_2^-$) and total phosphorus (P). The results in the table show that ammonia, nitrates and nitrites recorded values within the admitted limits for carp, while total phosphorus exceeded the admitted limit in the samples collected from the water draining point by approximately 2 times.

Phosphorus is the second most important chemical element from fisheries in producing organic substance, fish ponds productivity depending on it [7].

Iron, chlorine and sulfates belong to the general ions class of surface waters quality. The obtained values for iron and sulfates in all water samples range within the admitted limits for carps. Free chlorine recorded overvalues in all collecting points by approximately 10 times.

High chlorine concentrations added to a pH value beyond the optimal level for carp could lead to irritations and gills' damage.

The high chlorine concentration value revealed in the collected samples does not have a negative influence on fish life since the pH values are within the normal range and water temperature is under 20°C.

Of the metals group as surface waters quality indicators, copper was also assessed, which has recorded values up to the maximum admitted limit (0.3 mg/l) for carp.

Concerning the detergents (toxic substances - indicators of surface waters quality), it can be noticed that all the obtained values following the assessment are proper for carp rearing.

Water quality in Comisani fishery is adequate for carp rearing for most of the indicators, less free chlorine and it shows a high welfare level in relation with their living environment quality.

## 3. CONCLUSIONS

3.1. Among the water quality indicators, pH, dissolved oxygen, ammonia, nitrates, nitrites, iron, sulfates and detergents are proper for carp rearing.

3.2. Free chlorine has recorded overvalues in all collected samples by approximately 10 times.

3.3. Total phosphorus recorded an exceeding by approximately 2 times of the maximum admitted limits in samples collected from the water draining point.

3.4. Fish living environment from Comisani pond shows a good fish welfare.

*Acknowledgments.* This study has been financed and is a part of ID_285 grant CNCSIS UEFISCSU, contract Idei 290/2007: Researches concerning the welfare of fish in farm fish, in transportation and slaughtering units.

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# SUSCEPTIBILITY PROFILE OF MEDICAL IMPORTANT BACTERIAL STRAINS ISOLATED FROM MEAT AND DAIRY PRODUCTS

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**Key words**: antimicrobial resistance, *E. coli, S. aureus,* meat, dairy.

## SUMMARY

The aim of this study is to detect the prevalence of *Enterococcus faecium* and *Enterococcus faecalis, Escherichia coli* and *Staphylococcus aureus* strains resistant to usual antibiotics and risk assessment for its transfer on animal – food – human chain.
There was collected a total number of 123 (n=123), meat and dairy products samples from different commercial points from Iasi county. The samples were cultivated on enrichment broth and on selective agar, specific to each microorganism. After obtaining pure strains, these were identified using conventional methods. Thus, from the 90 dairy samples there were identified 17 strains of *E. coli*, two strains of *S. aureus*, 11 strains of *E. faecalis* and 13 of *E. faecium*. From the 33 meat samples there were identified two strains of *E. coli*, three strains of *S. aureus*, four strains of *E. faecalis* and three of *E. faecium*. Antimicrobial susceptibility degree was assessed for the isolated strains as recommended by CLSI standards. In the case of dairy samples, the antimicrobial susceptibility for both *E. coli* and *S. aureus* was 100% to all tested antibiotics. *E. faecium* strains isolated from dairy products presented a resistance ranging from 8% for ciprofloxacin and vancomycin, to 23% for penicillin and up to 85% to gentamicin. *E. faecalis* presented an increased resistance percentage for gentamicin (100%) and penicillin (27%). As for the meat samples, the *E. coli* strains presented an antimicrobial resistance percentage of 50% for amoxicillin, cafazolin, cefotaxime, ciprofloxacin and 100% for amikacin. The *S. aureus* strains from meat presented a resistance percentage of 33% for amoxicillin and clavulanic acid, ciprofloxacin and 67% for penicillin, ampicillin, clarithromycin. *E. faecium* from meat samples presented a resistance percentage ranging from 33,33% for penicillin and ciprofloxacin, to 66,66% for gentamicin and vancomycin. Yet, *E. faecalis* presented resistance only to linezolid (25%) and gentamicin (100%).

The injudicious use of antibiotics in veterinary field for livestock for both prophylactic and treatment purposes but also as weight promoters (outside European Union), increases the passage risk, for resistant bacteria, to humans by food chain. Continuing the awareness list addressed to clinicians, the injudicious use of antibiotics is considered to be the trigger force for the appearance of bacterial resistance (Kritsotakis and Gikas, 2006). Intensive farming systems led to also to a significant increase for the administration of antimicrobial agents to food producing animals. Any use of these antimicrobial agents represents a public health problem, due to development, selection and increasing incidence of

resistance and to the persistence of antimicrobial feed additives residues in food products of animal origin (Anadon and Martinez – Larranaga, 1999). Antimicrobial resistance may become a major problem in veterinary medicine as a consequence of the intensive use and especially of the misuse of antimicrobial drugs (Catry *et al.*, 2003). The repercussions in human health are represented by the appearance of multi-resistant food-borne pathogens and resistant microorganisms that infect animals and that produce illness to humans. There are studies that underline the fact that there are high rates of ciprofloxacin resistance in *E. coli* which can be associated with the use of fluoroquinolones in poultry. Latest researches in Europe brought out vancomycin-resistant enterococci with the vanA determinant, being believed to be due to the use of avoparcin (a glycopeptide antibiotic) used as growth promoter in food animals (Murray *et al*., 2003).

The main objectives of this study are the identification of the bacterial strains isolated from meat and dairy products samples using conventional kits and determining their susceptibility profile in conformity with CLSI standards.

## 1. MATERIAL AND METHOD

There were collected a total number of 123 samples (n=123), from which 33 samples were raw meat or meat products and 90 dairy samples (cow and sheep fresh cheese, cottage cheese, simple yogurt and fruit yogurt) from different commercial points from Iasi county. To be mentioned that all the samples were not overcoming the availability period and were kept in recommended conditions.

For the laboratory techniques were used special media, broth (nutritive broth and Lauryl sulphate broth) and agar (Baird Parker with egg yolk and telurite enrichment, TBX, Levine and Bile Esculin Azide agar), sterile swabs, thermostat, safety cabinet class II, rapid ID 32 STREP (bioMerieux, France) identification kit, human plasma, different antibiotics, with a specific concentration, belonging to A, B and C groups as recommended by CLSI standards (BioRad, France) and disk dispenser for the antibiogram.

The samples from meat and dairy products were collected with sterile swabs, then were cultivated on enrichment broth at 36±2°C 24 h for *Staphylococcus spp*. and *Enterococcus spp.,* and on Lauryl sulphate broth at 44,5°C 24 h for *E. coli*. After incubation period the samples were passed on selective agar, specific to each microorganism (Levine

for *E. coli*, Chapman and Baird Parker for *Staphylococcus spp.* and Bile Esculin Azide for *Enterococcus spp*.) also incubated at 36±2°C for 24 h.

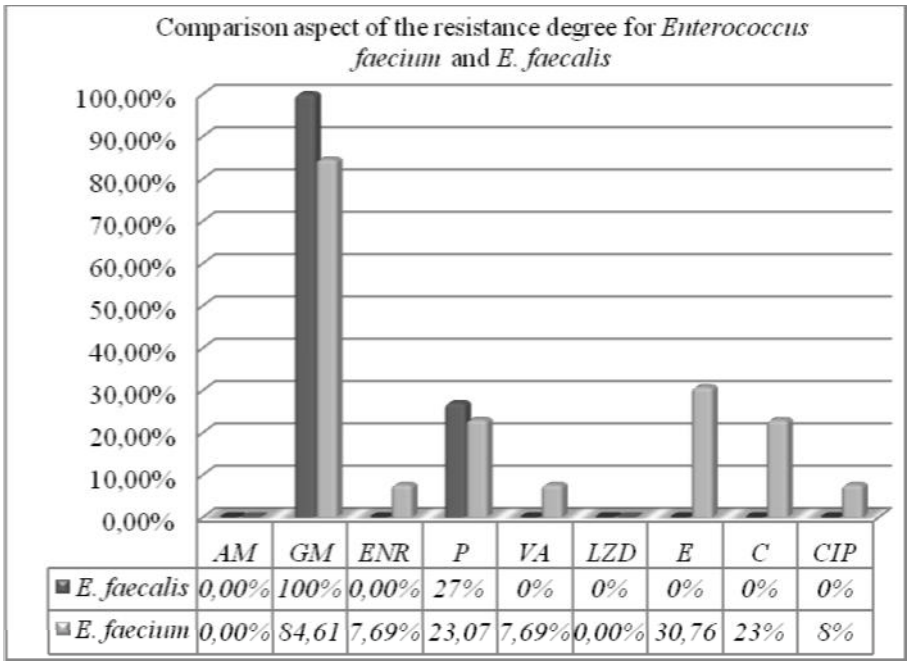
After obtaining the pure strains, these were identified to species level by using conventional methods. For *Enterococcus spp.* strains, Rapid ID 32 STREP was used (bioMerieux, France), a standardized system for the identification of streptococci and enterococci, and those most related organisms, in four hours, by using a specific database. Reading and interpretation were carried out manually. The principle of the methods is that the rapid ID 32 STREP strips consist of 32 test cupules which contain dehydrated test substances. After four hours of incubation at 36±2°C, the reactions are read visually and the identification is obtained using the identification software Api web™ (bioMerieux, Franţa). For *Staphylococcus spp.* was performed the citrated plasma coagulation test with human plasma, the results being noted after four hours of incubation at 37°C. Were selected only the strains which were coagulase positive. The *E. coli* can be distinguished from other coliforms by its growth and color reaction on certain types of culture media. Thus, when cultured on an EMB (Eosin Methylene Blue Agar - Levine's Formulation) plate, a positive result for *E. coli* is metallic green colonies on a dark purple media or blue color on TBX agar.

After selecting the bacterial strains, the antimicrobial susceptibility degree was assessed through Kirby-Bauer antibiogram method (or disk diffusion antibiotic sensitivity testing) (Schwalbe *et al.*, 2007) as recommended by CLSI standard M100 S18, 2008.

## 2. RESULTS AND DISCUSSIONS

After the identification of the pure bacterial strains, the results are further described. Thus, from the 90 dairy samples there were identified 17 strains of *E. coli*, two strains of *S. aureus*, 11 strains of *E. faecalis* and 13 of *E. faecium*. From the 33 meat samples there were identified two strains of *E. coli*, three strains of *S. aureus*, four strains of *E. faecalis* and three of *E. faecium*. Antimicrobial susceptibility degree was assessed for the isolated strains in conformity with CLSI standard M100 – S18, 2008. Therefore, each bacterial strain was considered susceptible, intermediary or resistant to a certain antibiotic.

In the case of dairy samples, the antimicrobial susceptibility for both *E. coli* and *S. aureus* isolated strains was 100% to all tested antibiotics.




**AM – ampicillin; GM – gentamicin; ENR – enrofloxacin; P – penicillin; VA – vancomycin; LZD – linezolid; E – erythromycin; C – chloramphenicol; CIP - ciprofloxacin.**

**Fig.1. Comparison aspects of resistance degree for E. faecalis and E. faecium, isolated from dairy products.**

*E. faecium* strains isolated from dairy products had resistance percentages as follows: 7,69% for enrofloxacin, ciprofloxacin and vancomycin, 23% for chloramphenicol and penicillin, 30,76% for erythromycin and 84,61% for gentamicin similar to results obtained in other studies made on bulk goat milk samples (Cortes *et al.*, 2006). *E. faecalis* presented an increased resistance percentage for gentamicin (100%) and penicillin (27%) in comparison with ampicillin, enrofloxacin, vancomycin, linezolid, erythromycin, chloramphenicol and ciprofloxacin to whose action it was susceptible (fig.1.).

As for the meat samples, the *E. coli* isolated strains presented an antimicrobial resistance percentage of 50% for amoxicillin-clavulanic acid, cefazolin, cefotaxime, ciprofloxacin and 100% resistance percentage to amikacin. *E. coli* was 100% susceptible to gentamicin, ampicillin, cefoxitin, imipenem, sulfamethoxazole and trimethoprim, ticarcillin, chloramphenicol and tazobactam and piperacillin (fig.2.).

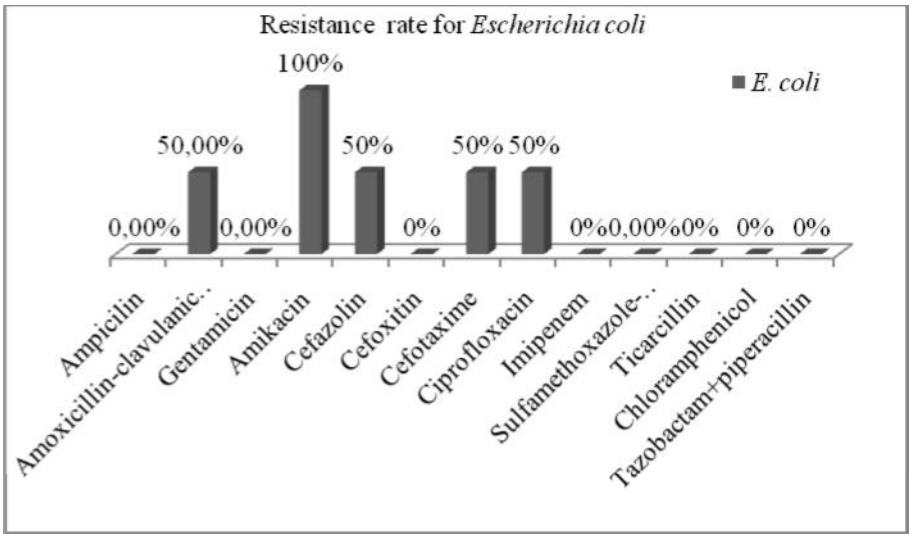



**Fig.2. Resistance for Escherichia coli isolated from meat products**

The *S. aureus* strains isolated from meat presented a resistance percentage of 33% for amoxicillin and clavulanic acid, clindamycin, ciprofloxacin and 67% for chloramphenicol, penicillin, ampicillin, and clarythromycine (fig.3.). The *S. aureus* strains are susceptible 100% to oxacillin and cefoxitin, which are MRSA indicators, in comparison with results obtained by Pasavento *et al*, from raw meat in 2007.

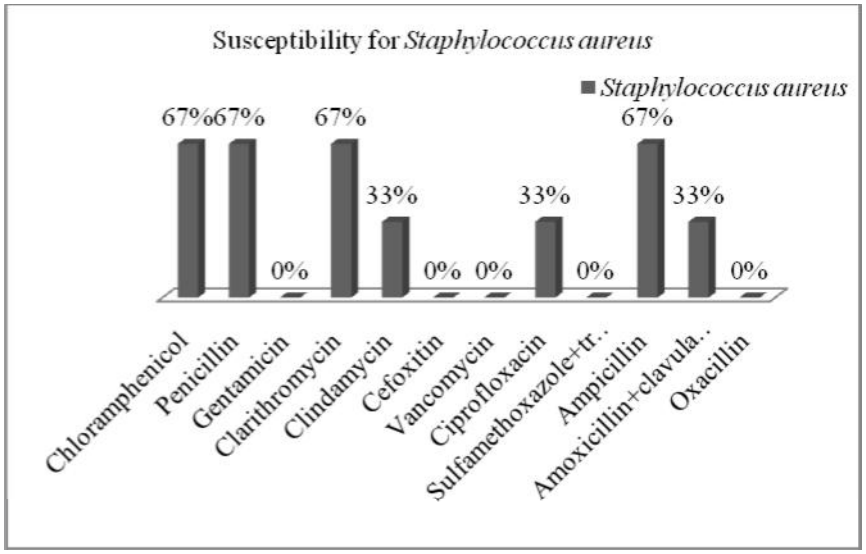
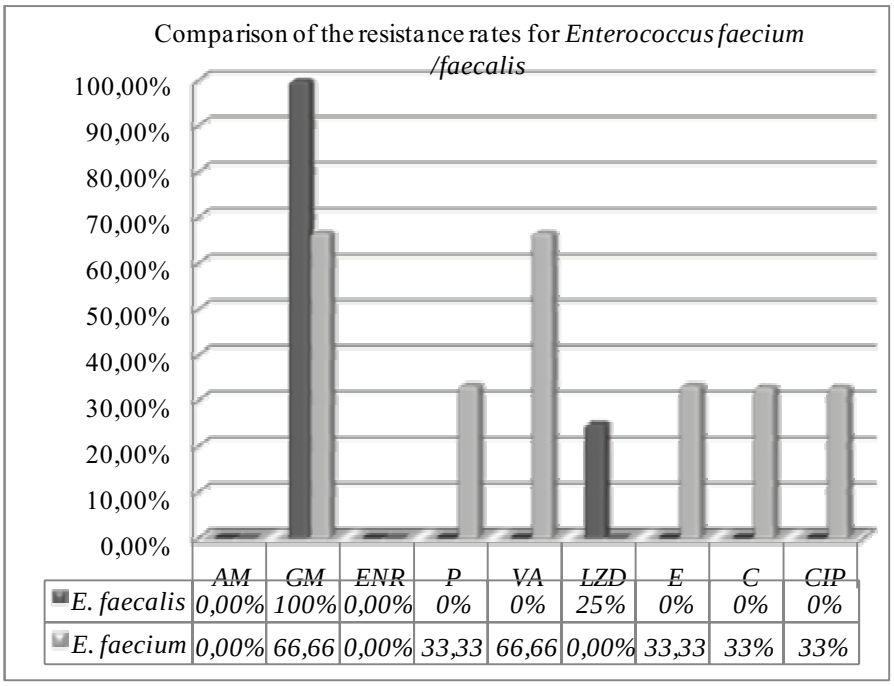



**Fig.3. Susceptibility for Staphylococcus aureus isolated from meat products**




| | AM | GM | ENR | P | VA | LZD | E | C | CIP |
|---|---|---|---|---|---|---|---|---|---|
| E. faecalis | 0,00% | 100% | 0,00% | 0% | 0% | 25% | 0% | 0% | 0% |
| E. faecium | 0,00% | 66,66 | 0,00% | 33,33 | 66,66 | 0,00% | 33,33 | 33% | 33% |

**AM – ampicillin; GM – gentamicin; ENR – enrofloxacin; P – penicillin; VA – vancomycin; LZD – linezolid; E – erythromycin; C – chloramphenicol; CIP - ciprofloxacin.**

**Fig.4. Comparison aspects of resistance degree for E. faecalis and E. faecium, isolated from meat products.**

*E. faecium* isolated from meat samples presented a resistance percentage ranging from 33,33% for penicillin, erythromycin, chloramphenicol, ciprofloxacin and 66,66% for gentamicin and vancomycin. *E. faecalis* isolated from meat samples presented resistance only to linezolid (25%) and gentamicin for 100% (fig.4.).

The obtained resistance of the isolated strains, towards the tested antibiotics, is considered to be due to antimicrobials used in animal feed, creating the possibility for both the antimicrobial residues and resistant organisms to contaminate the food and to be consumed by humans. Faye, K., 2007 – in the paper Current position in veterinary use of antibiotics: impact on antibiotic resistance of bacteria in animal and human health: underlines that the transfer of resistant bacterial strains from the animals treated incorrectly with antibiotics to humans, is possible through the food chain.

## 3. CONCLUSIONS

1.1. The obtained results indicate the fact that the antimicrobial resistance represents an emergence related to clinical or as growth promoters use of the antimicrobial agents, against which resistance is directed.

1.2. The obtained results emphasize the potential of these bacteria (*E. coli, S. aureus, E. faecium* and *E. faecalis*), to affect the human population through food chain (by consuming products of animal origin).

1.3. The resistance for *Staphylococcus aureus* and *E. coli* was mainly to the most common antibiotics used in therapy. Thus, *S. aureus* and *E. coli* exhibited resistance to β-lactam antibiotics (amoxicillin and clavulanic acid, ampicillin, penicillin), fluoroquinolones (ciprofloxacin), aminoglycosides and chloramphenicol.

1.4. The *S. aureus* strains isolated from meat products presents high resistance percentage to penicillin, ampicillin and amoxicillin and clavulanic acid, but they were susceptible to cefoxitin and oxacillin, fact that may indicate the presence of β-lactamases, but not meticillin-resistance (MRSA negative).

1.5. The presence of the bacterial strains isolated from products of animal origin resistant to some antibiotics, represents an extremely dangerous factor for public health.

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# CLINICAL AND THERAPEUTICAL STUDY IN EQUINE KELOID

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**Key words:** horse, keloid scar, teraphy

**SUMMARY**

The present study contain observation about the horse keloid scar concerning his appearance, clinical and therapeutically manifestation .In this purpose the authors took 18 horses in observation , the horses were pure and mixed Romanian horse breed .The keloid scar have predilection for posterior lambs .The size of keloid scar in variable and different from the size of a chestnut to the size of a hand ball. The keloid scar structure is based on collagen fibers, confirmed by the histopathology tests were it can be seen the anarchic distribution and big diameter of the collagen fibers. The keloid scar therapy is uncertain because the attempts with corticosteroids bandage (Contratubex crème),triamcinolone acetonid ) and silicone gel had uncertain results.
Good results were obtained after surgical treatment by ablation of the keloid scar, the skinless area was covered with skin and protected by a bandage. In cases of large keloid scars the results are inadequate because the diseases recur. In our casuistry we saw recur disease and complications in 4 cases .The complications were characterized by chronic *lymphangitis, chronic edema and skin sclerosis.*

The equine keloid is known by the excesses benign proliferation of dermal collagen following traumatic injuries.

The equine keloid is one of the most serious pathology of the wound scarring tissue, especially in horse, donkey and mule. Ragland W.L., Spencer G.R. (1970); Lane J.G. (1977) are mentioning that between the complete healing of the wound and the beginning of the keloid tissue may be a variable time line. Cotchin E. (1984) say that the equine keloid appears mostly after accidental wounds that are untreated or improperly healed.

This type of keloid is often localized at the limbs extremities (pastern, fetlock, shank, hock), where there is a poor muscular layer and a wealthy conjunctive tissue.

The keloid has a tumor-like shape and its structure is mostly type III and type II collagen, which has a fast growth both in horizontal and vertical pattern.

The aim of this study is to capture and to evaluate the beginning, the evolution and the therapy of this pathology which is a present times issue.

## 1. MATERIAL AND METHOD

Our observations were made during 10 years, on horses that arrived at the Surgery Clinic of the Veterinary Faculty in Cluj-Napoca to establish a diagnostics followed by proper therapy.

For this study were used 18 horses of different ages that were exploited for extensive work and also for sport. For each case the next aspects were recorded: size, localization, expansion, exterior aspect, tegument health status, presence and/or absence of necrotic tissue, presence of fistulas, regional changes due to the tumor compression, and patient's general health status.

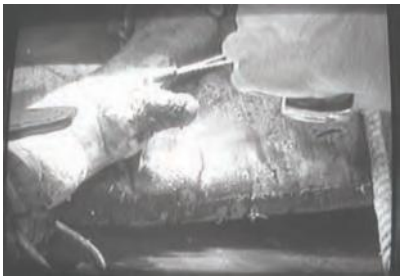
Regarding the therapeutic procedure it must be mentioned that all horses were submitted to preoperatory conduct before the surgical intervention.

The keloid ablation was made through the incision of the peritumoral tissues and penetrating with the scalpel at the base of tumor, and also keeping the marginal wound epithelium intact. The excision and the removal of the tumor was performed systematical, little-by-little, and keeping a good hemostasis. The hemostasis was made differently in each case by forcipressure, mediated suture, electro-cauterization, thermo-cauterization, and hemostatic buffers.

In cases where the tumors were small in size, the skin around the removed tumors was dilacerated and by mobilizing it the wound was closed using simple nods, by this manner the open surface that resulted after the tumor ablation was covered entirely by skin. Where the tumors were large in size the ablation process was performed by removing a little part each time, this was made to protect the surrounding anatomical structures (tendons, bones, nerves, sinovial sheath). After ablation topical therapy was applied for hemostatic and scarring purpose.

## 2. RESULTS AND DISCUTIONS

In our study, regarding the localization of the keloid, this was found in 85% of cases at the extremities of the limbs (metatarsal region, metacarpal region, pastern), mostly at the posterior limbs (Img.1. and Img.2.).



**Img.1. Fetlock localization**

**Img.2. Pastern localization**

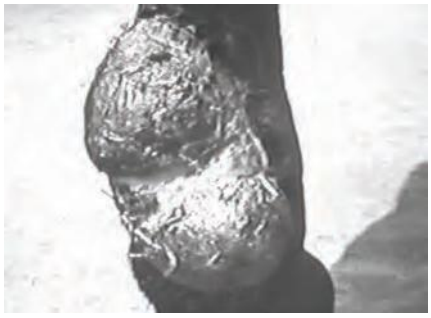
Next to the previous localizations, the tumors were found sporadically at the frontal (Img.3.) and cervical region, and anterior limbs.

**Img.3. Frontal region localization**

Other criteria that we followed in our research were the size of the tumors and their shape. The size of the tumors varied from the size of a nut to the size of a handball. The base of the tumors was either a pedicle or a large implantation area (Img.4.).

**Img.4. Large implantation area**

There was a large variability in what concerns the shape of the tumors, meaning that the most frequent shapes were: round, spherical, oval (Img.5.) and easy elongated. Due to this aspect in many cases the tumors have had a large implantation area.



**Img.5. Oval shape**

At the examination of the tissues surrounding tumors, the next aspects were encountered: reactivity, size enlargement, hardness, painless.

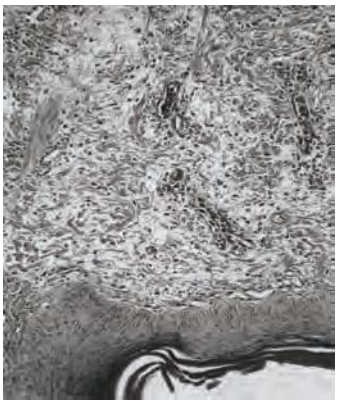
The aspects of the examined keloids were: the small ones had a smooth, glossy, red-pink surface, covered sometimes with dirt and/or hay; the large ones had an irregular cauliflower-like shape, with large creases, with a rough thick tegument, with debridements and with necrotic tissue and large deep fistulas. On the surface, some keloids were covered by dark crusts under which were necrotic areas with ulcers and suppurations, all these next to a disagreeable odor.

The presents and the growth of the tumors, in time, perturbed the venous blood flow, resulting edemas, limphangitis, which in time had led to dermal sclerosis etc.

In section, the aspect of the keloid is compact, dens, fatty (Img.6.), white-pink in color, with dotage areas to the surface and white in deeper aspect.

**Img.6. The dens appearance of the section**

Histological, the tumors appear as an agglomeration of collagen fibers, unorganized with a thick appearance in section (Img.7.).



**Img.7. The collagen fibers concentric pattern**

The hematological findings from horses with keloid were inconclusive. The surgical therapy was efficient in all 7 cases where the tumors size was little, the tissue gap being covered with the surrounded skin. In 4 cases, because of the large gap that resulted after the tumor removal, 20-25cm, the surgical intervention was not followed by success, even though next to it, topic medication with antibiotic powders, oxytetraciclin spray, antibiotic ointments or other substances were applied. It must be mentioned that more the surgical intervention is delayed more the skin structure changes by collagen infiltration, followed by less resistance of the skin. This will negatively modify the wound closure and its healing process.

## 3. CONCLUSIONS

3.1. The equine keloid appears in most cases next to unproper, incomplete or incorrect wound therapy.

3.2. The most frequent localization is at the posterior limbs, at the metatarsal, pastern and/or fetlock regions.

3.3. Clinically the tumor appears to vary in size, with or without complications, such as necrosis and dotage tumoral tissue.

3.4. The biological structure of the developed tissue composed from thick collagen fibers, with a circular and anarchic pattern.

3.5. The most efficient therapy is total ablation of the tumor and the gap closure with a skin fold.

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# THE HEMILAMINECTOMY INTERVENTION IN DOG MEDULLARY COMPRESION SYBDROM

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**Key words:** dogs, hemylaminectomy, treatemant

**SUMMARY**

In this study there have been made 18 surgical interventions of hemilaminectomy in dogs by different breed and ages. The medullary compression syndrome was installed suddenly in 16 cases and slows in 3 cases .The reasons of the medullary compression appearance were traumatic factors in the most of cases. The surgical treatment was the hemilaminectomy intervention, followed by physiotherapy, massage and swimming. The patient's recovery have been made between 20 and 65 days, but the footing and slow displacement started in day 15 in small dogs breed and in day 30 in large dogs breed.

The spine represents a complex anatomic structure with large variety of movements. The most used segment of the spin is the lombar region, and here the largest number of cases of medullary compression are found.

The medullary compression at this level can have different etiologies: disc protrusion, rahidian hemorrhage after vascular rupture, inflammatory response after disc prolaps or ligament rupture, disc degeneration, disc spondylosis etc.

Regardless of the hernia type (sudden or slow) this pathology represents a disease with an instant effect and also with serious consequences on the entire body.

## 1.MATERIALS AND METHODS

The study was made on 18 dog of different breeds and age, all presenting medullary compression syndrome. Next to clinic examination, complementary test were performed: radiological examination and mielografy.

At the clinic inspection the next aspects were encountered: hind limbs movement impossibility, hind limbs ataxia, hipo amyotrophy, seal posture. In some of the cases the patelar, gastrocnemian and tibial reflexes were present, and the other ones slowly diminished. In all patients with medullary compression syndrome diagnostics the

radiological findings confirmed the place and the factor of compression. In 15 cases the medullary compression syndrome developed suddenly, and in 3 cases it was slow. Due to continuous movements that the patients are doing for moving from one place to an other, the spine is susceptible to serious complications and even totally compromising its function. This being told we recommend the beginning of therapy after establishing the diagnostic.

In the cases where the compression factor is found (bone fragments, hematoma, disc prolaps) surgical therapy is advised.

The surgical therapy involved the dorsal hemilaminectomy technique, with preoperatory and postoperatory patient management.

The preoperatory preparation involved local hear clipping, aseptic and antisepsis management. The general anesthesia was obtained ether by neuroleptanalgesia, ether by gas anesthesia with isofluran.

The patient was placed in sterno-abdominal recumbency, the local anesthesia was made with xylasine or procaine, and then the surgical field was established.

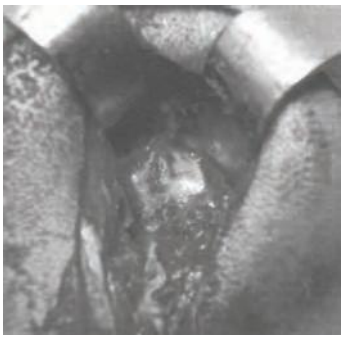
The surgical intervention begins with a dorso-median incision above the vertebral body, 4-6cm in length (Img.1.) depending on the size of the compression injury, and also not exceeding 2cm cranial or caudal from the affected region.

**Img.1. The incision**

The incision interests the skin, the subcutaneous tissue and the fatty tissue. The sectioned tissue is removed and the lombo-sacral fascia is incised next to the supraspinal ligament which it is close to. The tendon insertions of the *longissimus dorsi* muscle are sectioned at the level of the spinous processes and then the muscle is averted so that the spinous processes are free (Img.2.). This way the articular processes can be exposed so that the injured vertebra can be identified.

**Img.2. The section of the *longissimus dorsi* muscle**

With a special mill for osteotomy or with the Liston nipper the spinous processes and the vertebral arch are removed (Img.3.) until the dorsal lamina is reached. Then the dorsal lamina and the fatty tissue are removed with precaution so that the duramater is intact and the spinal cord stay intact and decompressed (Img.4.).

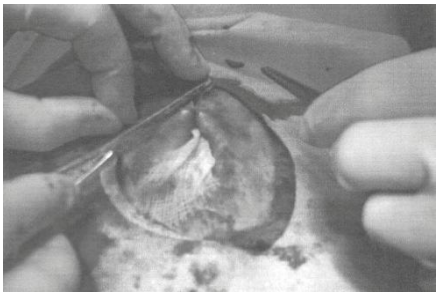


**Img.3. Vertebral arch appearance (bone lamina)**

**Img.4. The spinal cord after the vertebral arch is remove**

If there are splinters, clots or cysts, they will be removed with caution without injuring the spinal cord.

After decompression a safe hemostasis is assured, the surgical wound is washed with warm physiologic serum, the moisture is removed and a gauze drain (Img.5.) is placed at the surgical wound site to remove the exudate.



**Img.5. The simple points sutures at the skin level**

To establish the anatomical integrity of the region the *longissimus dorsi* muscul is freed and then sutured with resorbabal wire in simple points pattern, a pro cicatrisant powder is applied next, and then skin is closed with a simple points sutures.

## 2.RESOLTS AND DISCUTIONS

Postoperatory the patients were placed in lateral recumbency, on a soft and warm bed, so that they would be comfortable and the drainage was proper. In the first week the therapy included antibiotics and anti inflammatory drugs. In our case gentamicin 4mg/kc i.m. and dexametazone 1-2mg/10kg. Daily through the drain canal the surgical wound was washed with warm physiologic serum using a syringe, until the resulting liquid was free of fibrin clots.

5-7 days after surgery the patients began the physiotherapy and kinetotherapy with extensions and flexions 3 times per day, and also neuromuscular stimulants were administrated ($B_1$, $B_{12}$).

10-15 after surgery the patients were encouraged to stand up for at least 30-40 seconds in the first day, in time being able to stand for longer periods by themselves.

The results of this conduct are different, namely that in the small breed dogs (Teckel, Caniche, Cocker) the recovery was faster, while in the larger breed dogs the recovery was slower. Actually the patient involvement in the recovery process was mostly present in the small breed dogs.

In 5 cases, next to the procedure mentioned before, swimming therapy was applied beginning with the day 16 after surgery. The recovery in this cases was not faster (number of days) but there was a greater stability of the patient during walking. So 2 patients began to walk 20 days after surgery. The others began to walk between 20-65

days after surgery. The exception was a large breed dog (Rottwiller, male, 5 years old) in which case the recovery nedeed 90 days.

## 3.CONCLUSIONS

1. Hemilaminectomy may be a minimal invasive procedure compering to laminectomy in the medulary compression syndrome.
2. If the surgery is initiated earlier and at a short time after the trauma, the results will better.
3. The god postoperatory evolution is influenced directly by the means and the possibilities of recovery (physiotherapy, massage, felxions, extensions, swimming therapy).
4. The opening in the dorsal vertebral arch must be made with caution (to avoid the spinal cord trauma) and extended anterior and posterior only as much as it is necessary.

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# *IN VITRO* VIRUCID EFFICACY COMPARISONS OF MICROBICIDE PRODUCTS USED IN FARMS

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**Key Words:** virucid, microbicide product, microbial decontamination

**SUMMARY**

In present, evaluating of virulicid efficacy of microbicide products used in farms achieved in accordance with SR EN 14675:2006. In this work paper two commercially microbicide products used frequent in microbial decontamination of farms were comparative tested. The obtained results indicated that microbicide products were effective at 2% after 30 minutes contact time, different results comparatively with recommendations of manufacturer.

Romania Standards Association (ASRO), to 31 August 2006 adopted the European standard EN 14675:2006, which became SR EN 14675: 2006 to assess the virucidal activity of the disinfection substance used in veterinary field.

In the period 2005 - 2006, following outbreaks of avian influenza that have evolved in Romania, there was need for used the microbicide products with cid efficiency against avian influenza virus subtype H5N1 HPAI, which, along with other measures taken by authorities competent, avoid extension of disease outbreaks. Thus, since 2006, the Institute for Diagnosis and Animal Health (IDSA), the Major Viroses Department and High Containment Unit adopted SR EN 14675:2006 standard for assessment of virucidal effect of disinfection substance used in veterinary field, which used the virus isolation on cell culture technique, and bovine enterovirus type 1 (Picornaviridae), a small virus, nonanvelopated, with a high resistance in the environment and to microbicide products (5).

## 1. MATERIALS AND METHODS

*The materials* used were represented by: *Mardin Darby Bovine Kidney* cells (MDBK) from IDAH collection provided by Friedrich Loeffler Institute for inoculation and titration of virus. Cells were cultured in growth medium Eagle's minimum essential medium (MEM) supplemented with 1% antibiotics (penicillin 100UI/ml, streptomycin 100 mg / ml, mycostatin 100 mg / ml medium) and 10% fetal bovine serum; bovine enterovirus type 1 (ECBO), strain ATCC VR-248, lot no.

4095616 provided by American Type Culture Collection (ATCC) according to standard requirements, first passage of strain subjected of three times freezing and thawing, after growing on MDBK cell line, and centrifugation at 400 x g for 30 minutes to remove debris cellular. Virus titre calculated by Spearman and Karber method was 7.5 $\log_{10}$ TCID50/ml; 5% bovine albumin used like source of organic matter; two commercial microbicide products in five different concentrations (0.1%, 0.5%, 1%, 2%, 5%), from which three were recommended by the manufacturer and two were randomly selected at different contact times (1, 5, 10, 30 and 60 minutes) for each concentration, including contact time recommended by the manufacturer. Microbicide products have been represented by a product that has in composition peroxygen compounds and another product that has in composition iodine compounds.

*The method* is based on the presence or absence of cytopathic effect induced by bovine enterovirus type 1 in MDBK monolayer cultures cell after inoculation of the mix of microbicid product - virus. Evaluation of virucid effect of microbicide products was done in two stages: in the first stage has been performed *preliminary work*, according to the standard, which consisted of: preparation of antibiotic solution, mineralized water, albumin solution, trypsin-versen solution, suspension of MDBK cells, microbicide products solutions, virus suspension, titration of virus suspension, determination of cytotoxicity microbicide products, and in the second stage has been performed the *virucid evaluation* of mix of microbicide product – virus, according to standards, for each product microbicid at five contact times (1 minute, 5 minutes, 10 minutes, 30 minutes, 60 minutes) and for each concentration, and consisted of: preparation of the mix of product microbicid - virus, making decimal dilutions and distribution of dilutions in the microtitre plates, preparation and distribution of the cell suspension in the microtitre plates, incubation and examination at reversed microscope, reading and interpreting the results; performed the inactivation reference test which is a control of testing system using the formalin (37%), neutralizing the cytotoxic effect by ultracentrifugation of the mix of microbicid product - virus at 100 x G, 60 minutes and 4°C, remove supernatant, reconstituting of the pellets with 1 ml MEM supplemented with foetal bovine serum 2% and antibiotics 1%, transfer and distribution of cell suspension in microtitre plates (2).

## 2. RESULTS AND DISCUSSION

Evaluation of the virucid effect of microbicides products was done by calculating of the difference between the TCID50/ml $\log_{10}$ values of initially titres of virus and the $\log_{10}$TCID50/ml values of the titres of same virus obtained after action of microbicid product. A microbicide product was considered virucid efficacy if the results showed a reduction of viral titer $\geq$ 4 $\log_{10}$ $TCID_{50}$/ml. Cytotoxicity microbicide products has not been more than 1.0 $\log_{10}$, for 0.1% 0.5% and 1% solutions without neutralize by ultracentrifugation and for 2% and 5% solutions after neutralize by ultracentrifugation (2), results obtained were in limits accepted of the standard.

The positive control and monitoring viability of virus on MDBK cell line were normal positive (Figure 1).

Negative control and monitoring viability of MDBK cell line were normal negative (Figure 2).

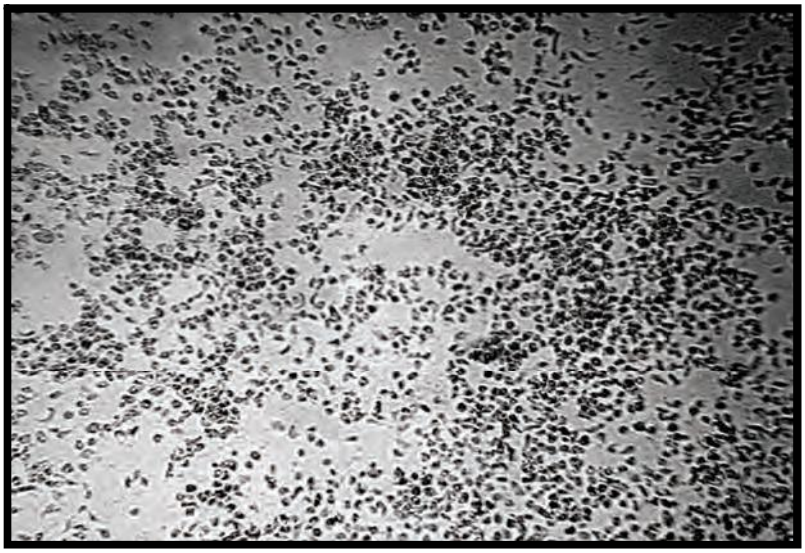

***Figure 1.*** **Generalized cytopathic effect in all cell line MDBK monolayer culture - round cells, refringente (positive control)**

***Figure 2*. Line MDBK cells without cytopathic effect (negative control)**

The ineficient cid effect of the commercial microbicide products tested against bovine enterovirus type 1 was evidenced by the presence of cytopathic effect (Figure 3).

*Table 1*

**The titer of bovine enterovirus type 1 before and after its contact with five concentrations of two microbicide products tested at five time periods**

| Microbicede product | Concentration of microbicide products / contact time (minutes)/ / bovine enterovirus type 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0,1% | | | | | 0,5% | | | | | 1% | | | | | 2% | | | | | 5% | | | | | |
| | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | **30** | **60** |
| peroxygen compounds | $10^{7,30}$ | $10^{7,15}$ | $10^{7,00}$ | $10^{6,30}$ | $10^{6,00}$ | $10^{7,00}$ | $10^{6,75}$ | $10^{6,30}$ | $10^{6,00}$ | $10^{5,75}$ | $10^{6,30}$ | $10^{6,00}$ | $10^{5,30}$ | $10^{4,75}$ | $10^{4,25}$ | $10^{5,30}$ | $10^{4,75}$ | $10^{3,80}$ | $10^{3,00}$ | $10^{2,00}$ | $10^{4,75}$ | $10^{4,00}$ | $10^{3,30}$ | $10^{2,50}$ | $10^{1,50}$ |
| iodine compounds | $10^{7,35}$ | $10^{7,25}$ | $10^{7,00}$ | $10^{6,50}$ | $10^{6,30}$ | $10^{7,00}$ | $10^{6,75}$ | $10^{6,50}$ | $10^{6,20}$ | $10^{5,75}$ | $10^{6,30}$ | $10^{5,75}$ | $10^{5,00}$ | $10^{4,75}$ | $10^{4,25}$ | $10^{5,30}$ | $10^{4,75}$ | $10^{4,00}$ | $10^{3,75}$ | $10^{3,00}$ | $10^{4,75}$ | $10^{4,00}$ | $10^{3,50}$ | $10^{2,75}$ | $10^{2,00}$ |

*Table 2*

**The difference between the initial titre of suspension of bovine enterovirus type 1 and titer of the same virus after the contact with five concentrations of two microbicide products tested at five times contact**

| Microbicede product | Concentration of microbicide products / contact time (minutes)/ / bovine enterovirus type 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0,1% | | | | | 0,5% | | | | | 1% | | | | | 2% | | | | | 5% | | | | | |
| | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 |
| peroxygen compounds | **0,20** | **0,35** | **0,50** | **1,25** | **1,50** | **0,50** | **0,75** | **1,25** | **1,50** | **1,75** | **1,00** | **1,50** | **2,20** | **2,75** | **3,25** | **2,20** | **2,75** | **3,70** | **4,50** | **5,50** | **2,75** | **3,50** | **4,20** | **5,00** | **6,00** |
| iodine compounds | **0,15** | **0,25** | **0,50** | **1,00** | **1,25** | **0,50** | **0,75** | **1,00** | **1,30** | **1,75** | **1,00** | **1,75** | **2,50** | **3,00** | **3,25** | **2,00** | **2,75** | **3,25** | **3,75** | **4,5** | **2,75** | **3,50** | **4,00** | **4,75** | **5,50** |

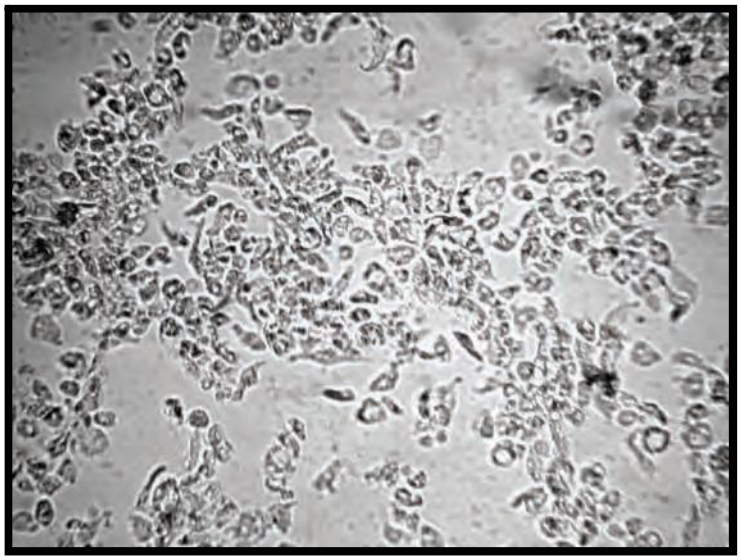

***Figure 3.*** **Generalized cytopathic effect - refringente and round cells**

The analysis of results presented in Tables 1-2 and Figure 3 shows that:

- commercial microbicide product tested, that was had in the composition the peroxygen compounds demonstrated the cid efficiency (titre reduction $\geq 4$ $\log_{10}$) to 2% after a contact period of 30 minutes, against bovine enterovirus type 1;
- commercial microbicide product tested, that was had in the composition iodine compounds demonstrated the cid efficiency (titre reduction $\geq 4$ $\log_{10}$) to 2% after a contact period of 60 minutes against bovine enterovirus type 1;
- commercial microbicide products tested, have not cid effect (titre reduction < 4 log10) at concentrations recommended by the manufacturers: 0.5% after 30 minutes contact time, for the product based on peroxygen compounds, and 1% after 30 minutes contact time, for the product based on iodine compounds. Manufacturers do not provide information of concentration, contact time and the enterovirus strain used in the testing of virucidal eficacy of their products;
- the results obtained in this study for microbicide products that contain peroxygen or iodine compounds were been similar with the results obtained by *Berg* et al., 2001, *Block* et al., 2001 and

*Chang* et al., 2004, without mentioned the enterovirus strain used to testing, and different with results obtained by *Yilmaz* et al., 2003, which reported the virucid efficacy at 0.5% after 15 minutes contact time.

To specify if the results are closer to the true value was used the parametric test of significance *t Student* (6), for a probability p = 0.05 (Table 3).

*Table 3*

**The *t* values for 7 degrees of freedom and a probability of error of 5% (p = 0.05) for the results obtained on cell culture in the testing of cid effect of two commercial microbicide products against bovine enterovirus type 1**

| Microbicede product | Concentration of microbicide products / contact time (minutes)/ bovine enterovirus type 1 | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0,1% | | | | 0,5% | | | | 1% | | | | 2% | | | | 5% | | | | | |
| | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | | |
| peroxygen compounds | 0,208 | 0,291 | 0,427 | 1,204 | 1,687 | 0,291 | 0,427 | 0,640 | 1,687 | 1,768 | 0,427 | 0,753 | 1,204 | 1,768 | 1,910 | 0,589 | 1,060 | 1,317 | 2,768 | 2,928 | 1,204 | 1,687 | 1,990 | 2,910 | 3,035 | |
| iodine compounds | 0,197 | 0,21 | 0,427 | 1,204 | 1,687 | 0,291 | 0,427 | 0,640 | 1,687 | 1,768 | 0,427 | 0,753 | 1,204 | 1,768 | 2,910 | 0,589 | 1,204 | 1,317 | 2,768 | 2,928 | 1,204 | 1,687 | 1,990 | 2,910 | 3,035 | |

The analysis of data presented in Table 3 shows that the *t* values calculated are greater than the *t* values from the tables for seven degrees of freedom (t = 1.895) and a probability of error of 5% (p = 0.05), indicating that the difference between virucidal efficacy and non virucidal efficacy is statistically significant and not due to sampling fluctuations.

## 3. CONCLUSIONS

3.1 Commercial microbicide products tested demonstrated cid efficacy against bovine enterovirus type 1, the results obtained in this paper recommending their use for microbial decontamination of the farms in solutions of 2% after 30 minutes and 60 minutes contact time.

3.2 No similar results were obtained with the concentrations and contact times recommended by the manufacturer (0.5% after 60 minutes contact time, for products based on peroxygen compounds and 1% after 30 minutes contact time, for products based on iodine compounds). Concentrations and contact times recommended by

manufacturers are similar to results reported *Ylamaz* et al., 2003.

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# EVALUATION OF THE CID EFFICACY OF THREE MICROBICIDE PRODUCTS AGAINST CLASSICAL SWINE FEVER VIRUS

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**Key words:** virucid, classical swine fever virus, microbicide products

**SUMMARY**

Studies have indicated variations in the degree of efficacy of the commercial microbicide products used in microbial decontaminating of the farms. In this work paper, three commercially microbicide products were tested against classical swine fever virus. The obtained results indicated that microbicide product which contained glutaraldehide and quaternary ammonium compounds were efficacy at 0,5% after 30 minutes contact time, microbicide product which contained quaternary ammonium compounds and surfactants were efficacy at 1% after 30 minutes contact time, and ethylic alcohol 70% was not efficacy.

Classical swine fever virus is a small virus, with a non-lipidic anvelope that confers resistance to lipophilic microbicide products (*Klein* et *DeForest*, 1983). To the evaluation of three microbicide products used a standardized method ASTM E1052-96 developed by the Environmental Protection Agency (EPA) of USA. In this study we changed, comparativ with the standard, the temperature of products evaluated, contact times, the number of determinations for each dilution and staining technique for virus identification. These changes were intended to facility the observation of the action of the factors considered to influence negatively the cid efficacy of microbicide products.

The method has been tested and validated within the Major Viroses Compartment and High Containment Unit from Institute for Diagnosis and Animal Health.

## 1. MATERIALS AND METHODS

The materials used were represented by: *PK-15 cell lines* from the collection of the IDSA, provided by the Friedrich Loeffler Institute and used for inoculation and virus titration. Cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 1% antibiotics (penicillin 100UI/ml, streptomycin 100 mg / ml, mycostatin 100 mg / ml medium) and 10% fetal bovine serum; classical swine fever virus strain (Romania/146/TM/07) subjected to freezing and thawing three times,

after growing on PK-15 cell line, and centrifugation at 400 x G for 30 minutes, at 4°C to remove cellular debris. Virus titre calculated by Spearman and Karber method and was 7.5 log10 TCID50/ml; conjugated anti- swine fever virus labelled with peroxidase; 5% foetal bovine serum as a source of organic matter; three commercial microbicide products tested at five different concentrations (0.1%, 0.5%, 1%, 2%, 5%), from which three concentrations were recommended by the manufacturer and two concentrations were randomly selected, and at different contact times (1 minute, 5 minutes, 10 minutes, 30 minutes and 60 minutes) for each concentration, including contact time recommended by the manufacturer. Microbicide products were represented by: 70% ethyl alcohol, a product that has in composition glutaraldehyde and quaternary ammonium compounds (QAC), and another product that has in composition surfactant and quaternary ammonium compounds (QAC).The method involves inoculating the mix of microbicid product - virus on PK-15 cell culture and evidencing of the infected cells *in vitro* with classical swine fever virus which has the ability to bind specific antiviral antibodies labeled with peroxidase. Evaluation of cid efficacy of the three microbicide products was done in two stages: the first phase were done the *preliminary work* consisted in preparation of: 1% antibiotic solution, trypsin-versen solution, albumin solution the cell lines suspension, viral suspension,, negative and positive controls, sodium acetate solution, wash buffer solution, dilution buffer solution, fixing solution, stop buffer solution, substrate solution, hyperimmune serum dilution, conjugate dilution and testing of the cytotoxicity of microbicide products (0.1 ml of A mixture were placed in 0.9 ml MEM medium supplemented 2% fetal bovine serum and 1% antibiotic), virus titration, and in the second stage was done the *virulicid evaluation* of each microbicid product at five contact times (1 minute, 5 minutes, 10 minutes, 30 minutes, 60 minutes) for each concentration and consisted in: preparation of product mixture microbicid – virus: first, preparing the *A mixture*: microbicid product and PBS (0.1 M) supplemented with 5% bovine albumin (source of organic matter), 1:5, and second, preparing the *mixture B*: one part of a viral suspension of work and 9 parts A mixture were prepared for five mixtures B and for five periods of contact (1 minute, 5 minutes, 10 minutes, 30 minutes and 60 minutes) and for each concentration of product microbicides, which were maintained at 10°C (± 1°C); the end of the contact time, from each mixture B were taken and were placed in 0.9 ml MEM medium supplemented with 2% fetal bovine serum and 1% antibiotics; decimal dilutions were made ($10^{-1}$ - $10^{-9}$), and remove 0.1 ml from each dilution and placed in 8 wells of

each microtitre plate, the last remaining 8 wells uninoculated with mix microbicid product - virus representing the cell control; distributing of 0.1 ml MDBK cell suspension (1.5 to 2 x 105 cells / ml) in MEM medium supplemented with 10% foetal bovine serum and 1% antibiotics in each well of the microtitre plates, inclusive in the last 8 wells reprezenting control cells; neutralize of the cytotoxic effect: ultracentrifugation the mix microbicid product - virus at 100 x G, 60 minutes and 4°C, remove supernatant, resuspension of virus in 1 ml MEM supplemented with 2% foetal bovine serum and 1% antibiotics, transfer and distribution of cell suspension in microtitre plates (2), incubation and examination to obtain monolayer: plates inoculated with mix of microbicid product - virus were incubated at 37°C and 5% $CO_2$ atmosphere for 2-4 days, examining daily until the cell monolayer formation; fixation for staining: liquid was removed from the plates and were introduced 150 ml of fixing solution to each well, which was eliminated after 1-2 minutes and was repeated distribution of 150 ml of fixing solution to each well, and then, the microtiter plates were incubated for 10 minutes to the room temperature (20$^{o}$C), which was removed after fixation solution; staining was performed by dispensing 50 ml conjugated anti swine fever virus labeled with peroxidase in all wells of microtiter plates, incubated for 60 minutes at 37°C in 5% $CO_2$ atmosphere, removal of the conjugate, washing with washing buffer, distributing of 50 ml substrate solution to all wells and incubated for 15 minutes under light and mixed. Stopping the colour reaction was achieved by adding 100 ml of sodium chloride solution 0.15% in all wells; reading and interpreting the results: reading is performed with a microscope reversed observing the presence or absence of cells red stained (due to virus multiplication in the cytoplasm). The presence of wells colored red constituted basis for calculation of virus titre (TCID50/ml) by *Spearman* and *Karber* method (negative log TCID50 = the smallest negative log dilutions - {[Σ (% positive / dilution) / 100] - 0.5} x log dilution factor). The results were expressed as $\log_{10}$ TCID50/ml. Evaluation of the virucidal efficacy of microbicide products tested was done by calculating the difference between the values of $\log_{10}$ TCID50/ml of initial suspension virus titre and values of $\log_{10}$ TCID50/ml of titre of same virus obtained after the action of microbicid products.

## 2. RESULTS AND DISCUSSION

A product has been considered virucidal efficacy, if the results showed a reduction in viral titer $\geq 4$ $\log_{10}$ TCID50/ml. The results are presented in Tables 1-3. Cytotoxicity of microbicide products has not been more than 1.0 $\log_{10}$ $TCID_{50}$/ml, for 0.1%, 0.5%, 1% solutions, without neutralize by ultracentrifugation, and for 2% and 5% solutions after neutralize by ultracentrifugation, the results were in the limits acceptable of the standard. The positive control and control of virus viability on cell line PK15 was normal positive (Figure 1). The negative control and monitoring viable cell line PK15 was normal negative (Figure 2).

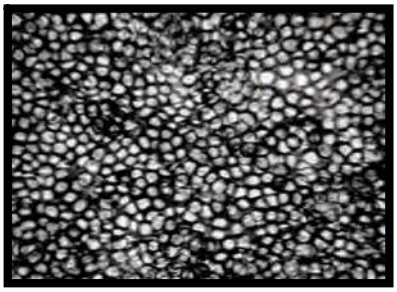

***Figure 1.*** **Cells colored red - brown indicating the presence of virus (positive control)**

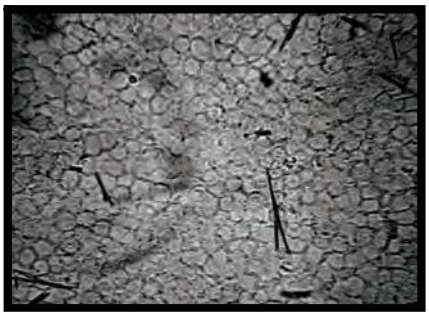

***Figure 2.*** **Uncolored cells in PK15 monolayer cell cultures uninfected with classical swine fever virus (negative control)**

*Table 1*

**The titer of classical swine fever virus before and after its contact with five concentrations of two microbicide products tested at five time periods**

| Microbicede product | Concentration of microbicide products / contact time (minutes)/ / classical swine fever virus | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0,1% | | | | | 0,5% | | | | | 1% | | | | | 2% | | | | | 5% | | | | |
| | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | **30** | **60** |
| QAC and glutaraldehidă | $10^{7,25}$ | $10^{7,15}$ | $10^{7,00}$ | $10^{5,75}$ | $10^{4,75}$ | $10^{6,15}$ | $10^{5,00}$ | $10^{4,30}$ | $10^{3,50}$ | $10^{3,20}$ | $10^{6,00}$ | $10^{4,75}$ | $10^{3,75}$ | $10^{3,00}$ | $10^{2,50}$ | $10^{5,20}$ | $10^{4,25}$ | $10^{3,5}$ | $10^{2,75}$ | $10^{2,00}$ | $10^{4,00}$ | $10^{2,75}$ | $10^{1,75}$ | $10^{1,00}$ | 0 |
| QAC and surfactants | $10^{7,25}$ | $10^{7,15}$ | $10^{7,00}$ | $10^{5,75}$ | $10^{5,00}$ | $10^{6,25}$ | $10^{5,75}$ | $10^{5,00}$ | $10^{3,75}$ | $10^{3,50}$ | $10^{6,15}$ | $10^{5,00}$ | $10^{4,3}$ | $10^{3,5}$ | $10^{2,75}$ | $10^{5,75}$ | $10^{4,75}$ | $10^{3,75}$ | $10^{4,2}$ | $10^{2,5}$ | $10^{4,75}$ | $10^{4,00}$ | $10^{3,00}$ | $10^{2,00}$ | $10^{1,00}$ |

*Table 2*

**The difference between the initial titre of classical swine fever virus and titer of the same virus after contact with five concentrations of two microbicide products tested at five times contact**

| Microbicede product | | Concentration of microbicide products / contact time (minutes)/ / classical swine fever virus | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0,1% | | | | | 0,5% | | | | | 1% | | | | | 2% | | | | | 5% | | | | |
| | | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 |
| QAC and glutaraldehidă | | 0,25 | 0,35 | 0,50 | 1,75 | 2,75 | 1,35 | 2,50 | 3,20 | 4,00 | 4,30 | 1,50 | 2,75 | 3,75 | 4,50 | 5,00 | 2,30 | 3,25 | 4,00 | 4,75 | 5,50 | 3,50 | 4,75 | 5,75 | 6,50 | 7,50 |
| QAC and surfactants | | 0,25 | 0,35 | 0,50 | 1,75 | 2,50 | 1,25 | 1,75 | 2,50 | 3,75 | 4,00 | 1,35 | 2,50 | 3,20 | 4,00 | 4,75 | 1,75 | 2,75 | 3,75 | 4,30 | 5,00 | 2,75 | 3,50 | 4,50 | 5,50 | 6,50 |

*Table 3*

**The evaluation of the cid efficacy of ethylic alcohol 70% against classical swine fever virus, based on the difference between the titre of initial virus and the titer of same virus after the microbicide product at five different contact times**

| Microbicide product | Concentration (%) | Contact time (minutes) | Titre virus after contact time $TCID_{50}$/ml | Initial titre of virus $TCID_{50}$/ml | Titre reduction after contact time |
|---|---|---|---|---|---|
| Ethylic alcohol | 70 | 1 | $10^{7,30}$ | $10^{7,5}$ | 0,2 |
| | | 5 | $10^{6,25}$ | | 1,25 |
| | | 10 | $10^{5,50}$ | | 2,00 |
| | | 30 | $10^{4,75}$ | | 2,75 |
| | | 60 | $10^{4,25}$ | | 3,25 |

The inefficient cid effect of commercial microbicide products tested against classical swine fever virus was evidenced by the presence of red-brown color of the infected cells (Figure 3).

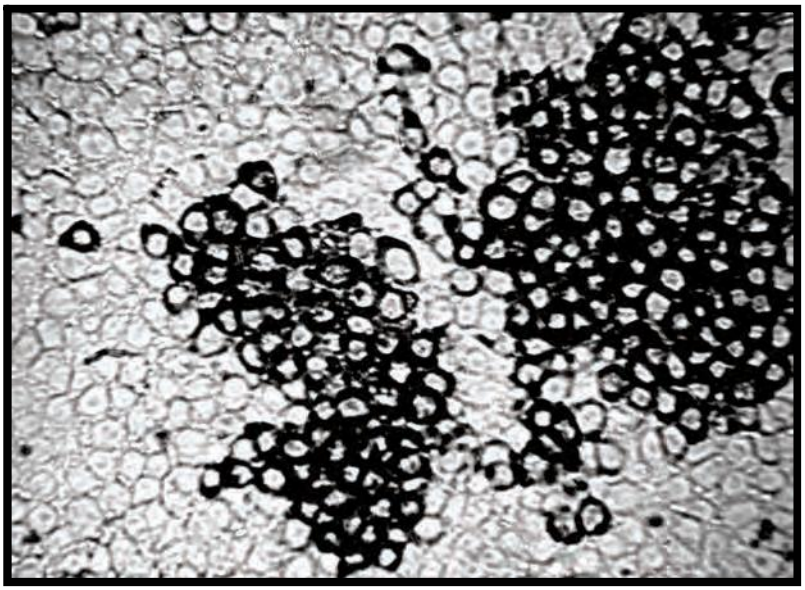

***Figure 3.*** **Cells stained red - brown indicating the presence of classical swine fever virus**

The analysis results presented in Tables 1-3 and Figure 3 shows that:

- the microbicide products that have in the composition glutaraldehyde and quaternary ammonium compounds had efficiency (reduction titre $\geq 4 \log_{10}$) against classical swine fever virus of: 0.5% and 1% after 30 minutes contact period, 2 % after 10 minutes contact period, 5% after 5 minutes contact period and did not efficiency (titre reduction $<4 \log_{10}$) of 0.1%, after all contact periods indicated by the test;
- the microbicid product which had in composition quaternary ammonium compounds and surfactants was efficiency (reduction titre $\geq 4 \log_{10}$) against classical swine fever virus of: 0.5% after 60 minutes contact period, 2% after 30 minutes contact period and 5% after 10 minutes contact period, and did not had efficiency (reduction titre $<4 \log_{10}$) of 0.1% after all contact periods indicated in test;
- 70% ethyl alcohol had no efficiency (reduction titre $< 4 \log_{10}$) against classical swine fever virus, after all contact period indicated by the test;
- the results obtained in this study for microbicide products that contain quaternary ammonium compounds and glutaraldehyde or quaternary ammonium compounds and surfactants are similar with the manufacturers recommendations (cid efficiency at 0.5 - 1% after 30 minutes contact time) and the results obtained by other researchers (Edwards et al., 2000);
- no existing available data regarding to the cid effect of 70% ethylic alcohol against classical swine fever virus.

To specify if the results are closer to the true value, was used the parametric significance test *t Student* (5), for a probability p = 0.05 (tables 4 and 5).

*Table 4*

**The *t* values for 7 degrees of freedom and a probability of error of 5% (p = 0.05) for the results obtained on cell culture in the testing of cid efficacy of ethylic alcohol against classical swine fever virus**

| Microbicide product | Concentrația % | Contact time (minutes) | *t* values |
|---|---|---|---|
| Ethylic alcohol | 70 | 1 | 0,736 |
| | | 5 | 0,836 |
| | | 10 | 1,000 |
| | | 30 | 1,687 |
| | | 60 | 1,801 |

*Table 5*

**The *t* values for 7 degrees of freedom and a probability of error of 5% (p = 0.05) for the results obtained on cell culture in the testing of cid efficacy of two commercial microbicide products against classical swine fever virus**

| Microbicede product | Concentration of microbicide products / contact time (minutes) / / classical swine fever virus | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0,1% | | | | 0,5% | | | | 1% | | | | 2% | | | | 5% | | | | | |
| | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 | 1 | 5 | 10 | 30 | 60 |
| QAC and glutaraldehidă | 0,208 | 0,291 | 0,427 | 1,204 | 1,687 | 0,291 | 0,277 | 0,640 | 1,901 | 2,768 | 0,427 | 0,753 | 1,204 | 2,768 | 2,910 | 0,589 | 1,260 | 1,901 | 2,768 | 2,928 | 1,204 | 1,901 | 2,768 | 2,910 | 3,035 |
| QAC and surfactants | 0,208 | 0,291 | 0,427 | 1,000 | 1,553 | 0,208 | 0,277 | 0,453 | 0,640 | 2,768 | 0,291 | 0,453 | 1,000 | 1,553 | 2,910 | 0,427 | 1,204 | 1,143 | 1,901 | 2,928 | 1,000 | 1,553 | 1,901 | 2,768 | 3,035 |

From analysing of data presented in Tables 4 and 5 resulted that the *t* values calculated are high than the *t* value from the tables for seven degrees of freedom (t = 1.895), and probability error of 5% (p = 0.05), indicating that the difference between virulicid efficacy and non virulicid efficacy of microbicide products is statistically significant and not due to sampling fluctuations.

## 3. CONCLUSIONS

3.1 Commercial microbicide products tested had cid efficacy against classical swine fever virus at concentrations and contact times recommended by the manufacturer ($p \geq 0.05$), except 70% ethyl alcohol, which had no cid efficiency ($p < 0.05$), after all the contact times estimated.

3.2 Personal research confirms the forecasts made by *Klein* and *DeForest*, 1983 regarding to the sensitivity of viruses with nonlipidic anvelope to the action of microbicide products.

3.3 The results confirm the seriousness of producers, by instructions for use with the concentrations and contact times accurate.

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# HERITABILITY ESTIMATION OF SOME CHARACTERS IN A LAYING HEN LINE BREEDING OBJECTIVE

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**Key words:** heritability, variance, genetic additive value, breeding value, phenotypic value.

**SUMMARY**

Heritability expresses the certainty of the phenotypic value as a guide of the breeding value or the correspondence range between the phenotypic value and breeding value. That is why the heritability is in the majority of the formulas used in animal breeding and several practical decisions depend on its size. Being ratio heritability is a feature belonging to a given character, and a given population [4]. Every heritability value is available to a certain population, in some circumstances. The correct management of the breeding work supposes its calculation in every given generation [3, 4].

The present researches proposed to achieve an estimation of the heritability, this being one of the most important feature of a character. It expresses the proportion of the total phenotypic variance which could be owed to the genes average effect, being defined as the ratio between the additive genotypic variance and the total phenotypic variance.

## 1. MATERIAL AND METHOD

The research in the present paper was achieved using a Leghorn hen line which formed the producing schemes of Albo 67 hybrid. The data come from 2007 generation and there were sampled from the production checking results in the whole line. The research was made on 80 male families (mix of sisters and half sisters) which consist of 1895 hens which end the eggs checking control. To present the genotypic, phenotypic and breeding parameters of the last studied generation (2007), there were sampled data regarding the following characters included in the breeding program of studied line:

1. number of eggs in the checking period (18-40weeks);
2. egg weight at 34 weeks;
3. body weight at 34 weeks;
4. age at first egg.

The phenotypic parameters for the studied characters are shown in table 1.

*Table 1*

**The phenotypic parameters in the retained hens in 2007 generation**

| No. | Character | Average and its error |
|---|---|---|
| 1 | Number of eggs | 99,82±0,58 |
| 2 | egg weight at 34 weeks | 57,73±0,07g |
| 3 | Body weight at 34 weeks | 1488,7±7,1g |
| 4 | age at first egg | 126,3±0,29days |

The experimental plan of this research supposes at the beginning the variance analyze with three variation sources (fathers, mothers, descendants) in a non-balanced hierarchic model (different number of descendants per mother and different number of mothers at the same father).

To establish the heritability it was used the causal components of variance method knowing that the heritability is defined as the ratio between the additive genetic variance (breeding value variance) and the total phenotypic variance:

$$h^2 = \frac{V_A}{V_F}$$

Knowing the fact that :

$$V_A = 4S^2_{FATHERS}$$

It is made the ratio $V_A/V_{F,}$ so it is obtained the value of the coefficient of heritability.

The precision of heritability estimation was calculated using the simplified formula of Robertson (quoted by Popescu-Vifor St.):

$$S_{h^2} = \left( h^2 + \frac{4}{n_i} \right) \sqrt{\frac{2}{s}}$$

Where:

$n_i$= the average number of descendants of the families;

s= number of families.

The heritability is calculated as:

$$h^2 = \frac{V_A}{V_{Total}} = \frac{4S^2_{FATHERS}}{V_{Total}}$$

## 2. RESULTS AND DISCUSSIONS

The correct management of the whole breeding process, as also the whole selection activity of laying hens suppose a very correct assessment of the studied characters heritability, this being the factor of precise decisions taken by the breeder and also the time necessary to achieve the proposed objectives.

In the table 2 there are shown the observational components of variance for the studied characters, the necessary elements to assess the values of heritability and their errors for the five characters in the study.

By the results in table 2 regarding the variance between fathers and the total variance there were estimated the values of the heritability coefficients for the studied characters, values shown in table 3.

Analyzing the results presented in table 3 regarding the value of the heritability coefficients of the studied characters, as the first observation it may say that these are comparable with the data in the special literature and they could frame the studied characters in two categories:

- intermediary characters, where the age at the first egg is framed as value ($h^2$=0,231)
- low heritable characters, where the number of eggs is framed ($h^2$=0,098) the egg weight at 34 weeks ($h^2$=0,124), and the body weight at 34 weeks ($h^2$=0,170). Comparing the obtained values in the present paper with the ones met in the special literature we could notice the following:

- Regarding the heritability of the number of eggs ($h^2$=0,098), Henderson and King obtained $h^2$=0, 31, Merrit found $h^2$=0,17( quoted by Gh. Sandu, 1983), Betianu in 1995 obtained $h^2$=0, 24, and I. Neagu, in 2009, $h^2$=0, 036.
- Regarding the heritability of body weight at 34 weeks ($h^2$=0, 170), Lerner and Cruder, (quoted by Sandu in 1983) found a value of $h^2$=0, 2, Betianu in 1995obtained $h^2$=0, 35 and I. Neagu in 2009, $h^2$=0, 226.

- Regarding the egg weight, $h^2$=0, 124, it recorded variations in different studies: Betianu (1995) obtains $h^2$=0, 220, I. Neagu (2009), $h^2$=0, 214.
- Regarding the heritability of the age at the first egg, $h^2$=0, 231, it recorded a higher value beside the existent values met in the special literature.

***Table 2***

**The observational components of variance in the studied characters**

| Components of variance | No of eggs | egg weight at 34 weeks | Body weight at 34 weeks | age at first egg |
|---|---|---|---|---|
| Between fathers | 238,49 | 310,82 | 422,17 | 348,08 |
| Between mothers at the same father | 126,58 | 622,15 | 1538,11 | 249,89 |
| Between descendants at the same mother | 9405,32 | 9062,35 | 7973,16 | 5435,43 |
| Total | 9770,39 | 9995,32 | 9933,44 | 6033,40 |

***Table 3***

**The values of the studied characters heritability and their error**

| No. | Character | Values of heritability and its error |
|---|---|---|
| 1 | Number of eggs | 0,098±0,035 |
| 2 | egg weight at 34 weeks | 0,124±0,042 |
| 3 | body weight at 34 weeks | 0,170±0,049 |
| 4 | age at first egg | 0,231±0,057 |

## 3. CONCLUSIONS

3.1. The correct management of the breeding work proposes the assessment of the genetic parameters in each generation of the population which work with. Every comparison with data in the special literature is absolutely directional. So, even in the quoted examples it may notice a large variability.

3.2. The values of the heritability coefficients estimated in the present paper for the age at the first egg ($h^2$=0,231), placed this feature in the intermediary transmitting features category.

3.3. The values of the estimated heritability coefficients for the number of eggs ($h^2$=0,098), the eggs weight ($h^2$=0,124) and for the body weight ($h^2$=0, 170) are placed within the low heritable characters, having in the analyzed population a low genetic determinism.

3.4. The values of the estimated heritability coefficients allow the choice of the selection method, in this case it is imposed the combine selection applying, knowing that in moderate and low heritable characters the main contribution in breeding value assessment is given by the family average, especially the father family average, and the own performances have a low support.

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# ORGANIC LESIONS INDUCED BY MYCOTOXINS THAT HAVE BEEN PRODUCED BY SOME FUNGI ISOLATED FROM FOOD

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**Keywords:** Aflatoxin, ochratoxin, injuries, fungi, laboratory mice.

**SUMMARY**

Mycotoxins are important pathogens because are mostly produced in large feed storage substrates, where fungi that produce those toxins find optimum conditions for development and their presence in food and animal feed is a matter of national interest. Among the species of fungi producing mycotoxins include those of the genera Aspergillus, Penicillium, Fusarium and Cladosporium. The most known toxins are: aflatoxin, ochratoxin, zearalenone, ergotamine and deoxynivalenol.
In the present study was used as matrix bread, bakery products, cheese and wheat, raw materials for bread production. Since the values of CFU / g product have exceeded the maximum allow values according the orders nr. 975/1998 and 976/1998, have led us to experience the effects of these toxins produced by mycetes on living organisms.To highlight the damage caused by these mycotoxins were inoculated white laboratory mice with 2.5 ppm and 4.5 ppm ochratoxin and total aflatoxin.
Ingestion of food containing mycotoxins can cause serious adverse effects on animals and consequently to human health.

Fungal contamination may result from air, from soil, or through insects, which can be vectors or by damaging the cereals' crust, as they are facilitating fungal development. (Palmgren M.S., Lee L.S., 1986) Mycotoxins occurrence may be influenced by different environmental factors, the contamination being different from one geographic area to another, while fungus development and the contamination with the toxins they produce will be the consequence of fungus-host-environment interaction. (Angus F.,1998)

The occurrence of Mycotoxins in food and animal fodder represents a matter of national interest. Ingestion of food containing Mycotoxins, secondary toxic byproducts of certain microscopic fungi, may determine serious adverse effects upon humans and animals health status. (Potroviță M., *et* al., 2008)

They may be present in the fodder, as well as in foodstuff (eggs, meat, milk, grains and cereals) under certain conditions of temperature and moisture. Mycotoxins have a negative action, especially upon the vital organs (heart, kidney, and liver) and blood.(Eaton, D. and E.P. Gallagher**,**1993) They came to be considered a risk factor both for human and for animal health, heaving been proven their presence in both animal and vegetal byproducts in amounts dangerous for public health. (Coman, I., and O.Popescu,1985)

## 1. MATERIALS AND METHODS

For this study we used as matrix potato bread, bread products (bagels, buns with sesame seeds), cow cheese and wheat used as raw material in baking industry. The culture medium used for mycological study of these products was DDCA (dextrose, glucose-isomer dextrogir, cloranfenicol and agar) and peptone water.

From the resulting mixture from each product that we tested for the presence of fungi, we take about 10 grams of sample and add 90 ml peptone water. Then we made serial dilutions in peptone water ($10^{-2}$and $10^{-3}$). From each dilution were seeded for each product, two Petry plates each with medium DDCA.

After being incubated at 27$^{o}$C, the plates were analyzed both in terms of macroscopic and microscopic point of view. Then we set the total number of fungi/gram of product, expressed as colony forming units (CFU). To determine the types of the fungi, we made preparations between the slide and microscope slide, using the lactofenol mount.

Since the species identified exceed the amount permitted by 975 and 976/1996 standards, which is 100UFC/g, thus being highly toxigenic, laboratory mice have been inoculated with toxins produced by certain species of these fungi, aflatoxin and ochratoxin to watch the impact of mycotoxins on internal organs

Doses of 2.5 or 4.5 ppm Aflatoxin and Ochratoxin have been injected and 14 days after inoculation, we take samples from liver and kidney and made histological preparations using tri chromic method.

## 2. RESULTS AND DISCUTIONS

Following the present research work the following results have been obtained, systematized in the table below:

***Table 1***

**Species of fungi obtained in our study**

| Product reviewed | Species of fungi | UFC/g |
|---|---|---|
| potato bread | *Penicilium expanseum, Aspergillus verzicolor, Aspergillus niger* | 4,9 x $10^3$ |
| bread products | *Aspergillus verzicolor, Penicilium expanseum, Fusarium roseum* | 3,4 x $10^3$ |
| cow cheese | *Penicilium expanseum, Cladosporium* | 7 x $10^3$ |
| wheat used as raw material in baking industry | *Aspergillus verzicolor, Penicilium expanseum, Penicilium claviforma Fusarium roseum,Rizopus stolonifer* | 4,1 x $10^3$ |

Analyzing the microscopic samples several types of mycets have found.

**Fig.1. *Penicillium expansum* X40, lactophenol mounting.**

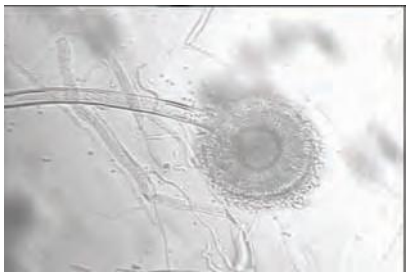

**Fig.2. *Aspergillus niger* X40 lactophenol mounting.**

Species of the *Penicillium* genus have been identified represented by *Penicillium expansum* and *Penicillium claviforma* in the following products: bread with potatoes and wheat grains as raw material for bakery products.(fig.1) Species of the genus *Asprgillus* have been identified in products like: bread with potatoes, pastry product and wheat grains as raw material for bakery products. (fig.2)

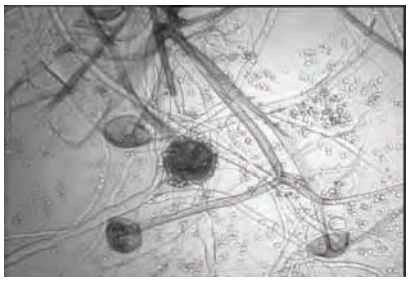

**Fig.3. *Rhizophus stolonifer* X40, lactophenol mounting**

**Fig.4. *Fusarium roseum*.X40, lactophenol mounting**.

Fungi from the genus *Rhizophus* and *Fusarium* have been identified in bakery products and wheat grains as raw material for bakery products. (fig. 3, 4)

At the same time, in the present study modifications over the liver and the kidney have been studied following the administration of Aflatoxins and Ochratoxins in white lab mice, in different doses (2,5ppm and 4,5 ppm), to demonstrate the dangerous effects of those toxins over living beings.

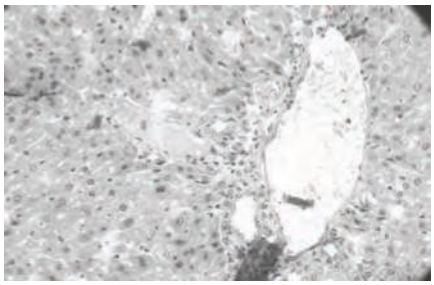

**Fig.5. Liver after administration of 2 ppm of Aflatoxin. (H.E.BM.,X 20)**

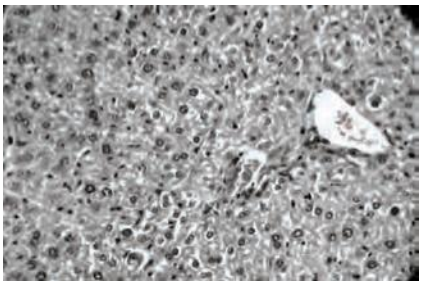

**Fig.6. Liver after administratio of 4 ppm of Aflatoxin. (H.E.BM., X 40)**

Thickening of the centrolobular vein intima has been noticed, as well as modification of the hepatic lobule structure, with migration of the centrolobular vein close to the Kiernan space. An area of edema can also be noticed, with absorption vacuole, and the background of portal circulation impairments, thickening of the billiary ductules and the presence of parietal thrombi in the hepatic arteriole. (fig.5) Congestion of the sinusoid capillaries was noticed, as well as strong connective tissue reaction (fibrosis) when we injected Aflatoxin. (fig.6)

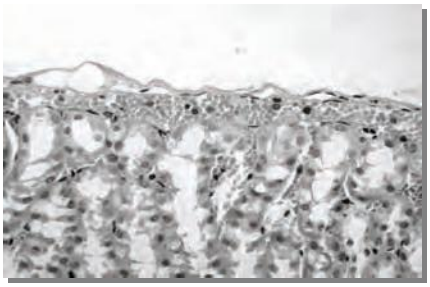

**Fig.7. Kidney after administration of 2 ppm of Aflatoxin. (H.E.BM., X 20)**

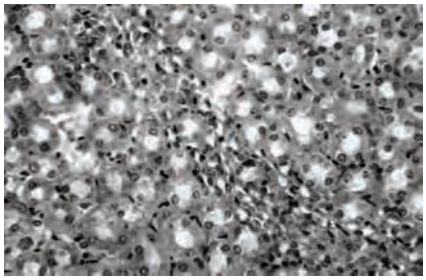

**Fig.8. Kidney after administration of 4 ppm of Aflatoxin. (H.E.BM., X 40)**

Massive necrosis of the renal tubules from the cortex, together with blood capillaries congestion and thickening of the renal capsule with decapsulation. (fig.7) Sub capsular hemorrhages were noticed, as well as congestion of the blood capillaries and compression atrophy of the renal tubules in the renal medulla. (fig.8) (Badea, M, et al, 2009)

After we injected Ochratoxin, an area of edema with absorption vacuole can be noticed, with thickening of the vascular wall intima. Perivascular limpho-histiocitary infiltration was also noticed, as well as different phases of necrobiosis affecting the hepatocytes. (fig.9)

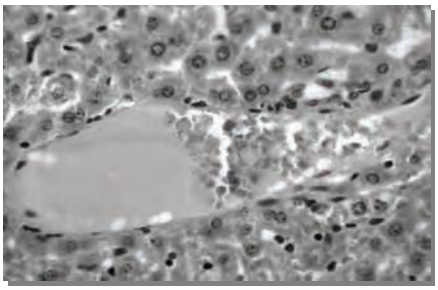

**Fig.9. Liver after administration of 2 ppm of Ochratoxin. (H.E.BM., X 20)**

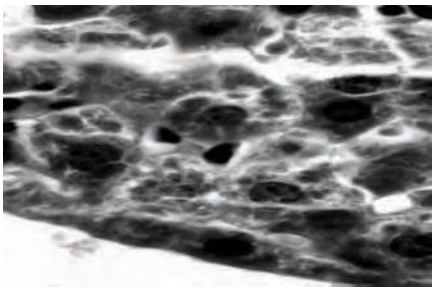

**Fig.10 Liver after administration of 4 ppm of Ochratoxin. (H.E.BM., X 40)**

At the same time, a lot of dystrophic hepatic cells were noticed, with area of necrosis, massive congestion of the sinusoid capillaries and the presence of numerous lymphocytes. (fig.10)

**Fig.11. Kidney after administration of 2 ppm of Ochratoxin. (H.E.BM., X 20)**

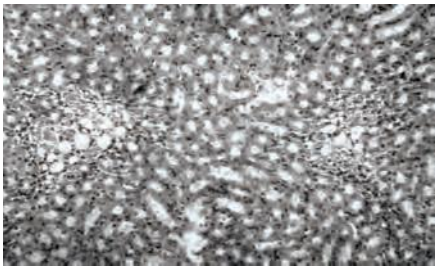

**Fig.12 Kidney after administration of 4 ppm of Ochratoxin. (H.E.BM., X 40)**

The presence of many atypical cells represented by large cells with intense basophilic cytoplasm, with the nucleolus eccentrically placed and a halo around the nucleus, as well as the evidence of adipose cells, among which modified renal tubules were noticed and multiple congestive capillaries, aspect that pleads for the evidence of certain proliferative lesions in the renal medulla. (fig.12) (Potroviță M., *et* al., 2008)

## 3. CONCLUSIONS

3.1. Following the present study, we noticed a massive fungal infestation of different alimentary products. No direct correlation existed between the total number of expressed fungi and the organoleptic traits of the products.

3.2. Depending on the administered dose Aflatoxins generate proliferative lesions in the liver, at the level of the blood vessels, producing serious circulatory impairments, modifications of the hepatic lobular structure, hemorrhagic lesions and even cirrhosis, in extreme cases. In kidney, Aflatoxin generates decapsulation, dystrophic lesions and necrosis and, at higher doses, cortical sclerosis.

3.3. With Ochratoxin, proliferative congestive lesions and circulatory disturbances appear in the liver, with extensive hepatic dystrophy. At higher doses, centro-lobular necrosis and sinusoidal congestion are generated. In kidney, Ochratoxin generates progressive decapsulation and, at higher doses, determines the production of vascular masses and cellular irregularities, possibly carcinomas.

3.4. They are, thus, sufficient for the evidence of those toxins that have very dangerous effects over animal and human health.

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# INFLUENCE OF PLATING MEDIA ON DETECTION, NUMERATION AND SPECIES OF *CAMPYLOBACTER SPP.* FROM NATURALLY CONTAMINATED CHICKEN SAMPLES

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**Key words**: *Campylobacter*, selective media, agar, chicken, growth.

**SUMMARY**

In the European Union, campylobacteriosis are the most frequently reported foodborne illnesses in humans. Broiler meat is considered to be an important food-borne source of these human diseases.
The purpose of this study was to determine which plating media has the best productivity using three different techniques of inoculation. There were tested 78 samples represented by neck skin; the plating media were mCCD agar, Preston agar, Butzler agar and Karmali agar.
This study revealed that *Campylobacter* numeration is dependent on the used medium: Butzler agar presented the lowest productivity, while between Preston and mCCD agar no significant differences were observed.

Globally, *Campylobacter* has been recognized as a leading cause of human gastroenteritis, generating considerable interest in the development of special selective technique for optimal growth and isolation. *Campylobacter* is a microaerophilic microorganism, sensitive to natural levels of oxygen found in the environment, thus requiring specific conditions for growth.

The methods described herein apply a microaerophilic atmosphere completed with supplements such as blood, charcoal, ferrous sulfate and sodium pyruvate, which are though to act by quenching toxic oxygen derivatives that develop over time in the media.

The purpose of this study was to evaluate different cultivation media for isolation of *Campylobacter spp.* from neck skin samples collected from broilers.

## 1. MATERIALS AND METHODS

Several selective media containing a different composition are available for culturing thermophilic enteropathogenic campylobacters from broilers. In this study we compared four plating media which differ both in basic composition and added antibiotic supplements; thus, there were chosen Karmali agar, Butzler agar, mCCD agar and Preston agar.

There are used various base formulations to which different antimicrobial and growth supplements can be added for selectively culturing the organisms on solid media (Oyarzabal *et al.*, 2005).

Some media contain whole sheep blood as Butzler agar, whereas defibrinated lysed horse blood is the choice of Preston agar and Skirrow agar. In other media, the blood was replaced with sodium pyruvate, ferrous sulfate (mCCD agar), hematin (Karmali agar) and charcoal (mCCD agar, Karmali agar). All these components are use to promote the growth of *Campylobacter* species, as they quench the toxic forms of oxygen (hydrogen peroxide), increasing the aerotolerance and enabling the oxygen sensitive strains to be readily isolated (Adley, 2006).

A combination of antibiotics is usually added to inhibit competing bacteria present in samples. Sodium deoxycholate partially or completely inhibits Gram positive organisms, coliforms and *Proteus*. Yeast and fungal contaminants are inhibited with the addition of amphotericin B (Scherer *et al.*, 2006, Washington *et al.*, 2006).

The supplements of antibiotics added in the studied culture media are presented in table 1.

***Table 1***

**The composition in antibiotics of the media**

| **Antibiotic supplement** | **Plating media** | | | |
|---|---|---|---|---|
| | **Karmali agar** | **Butzler agar** | **mCCDA agar** | **Preston agar** |
| Vancomycin | x | - | - | - |
| Cefoperazone | x | x | x | - |
| Rifampicin | - | x | - | x |
| Colistin | - | x | - | - |
| Amphotericin B | - | x | x | - |
| Polymyxin B | - | - | - | x |
| Trimethoprim | - | - | - | x |

Food samples that were used were represented by neck skin taken from chicken carcasses. There were analyzed 78 samples noted from 1 to 78, according to ISO 10272-1,2/2006 standard. The procedure diagram is presented in the fig 1.

Over 10 grams of sample, there were added 90 ml of Bolton broth, and then there were made decimal dilutions. The solid selective media were inoculated with 0.1 ml of the initial and decimal dilutions. The plates were incubated at 41.5°C in micro-aerobic atmosphere for 48 hours. The colonies presumed to be *Campylobacter* were subcultured on

the non-selective agar medium, Columbia blood agar, and then they were confirmed by microscopic examinations and biochemical and growth tests. The number of *Campylobacter* / gram sample was calculated from the number of confirmed typical colonies / plate.

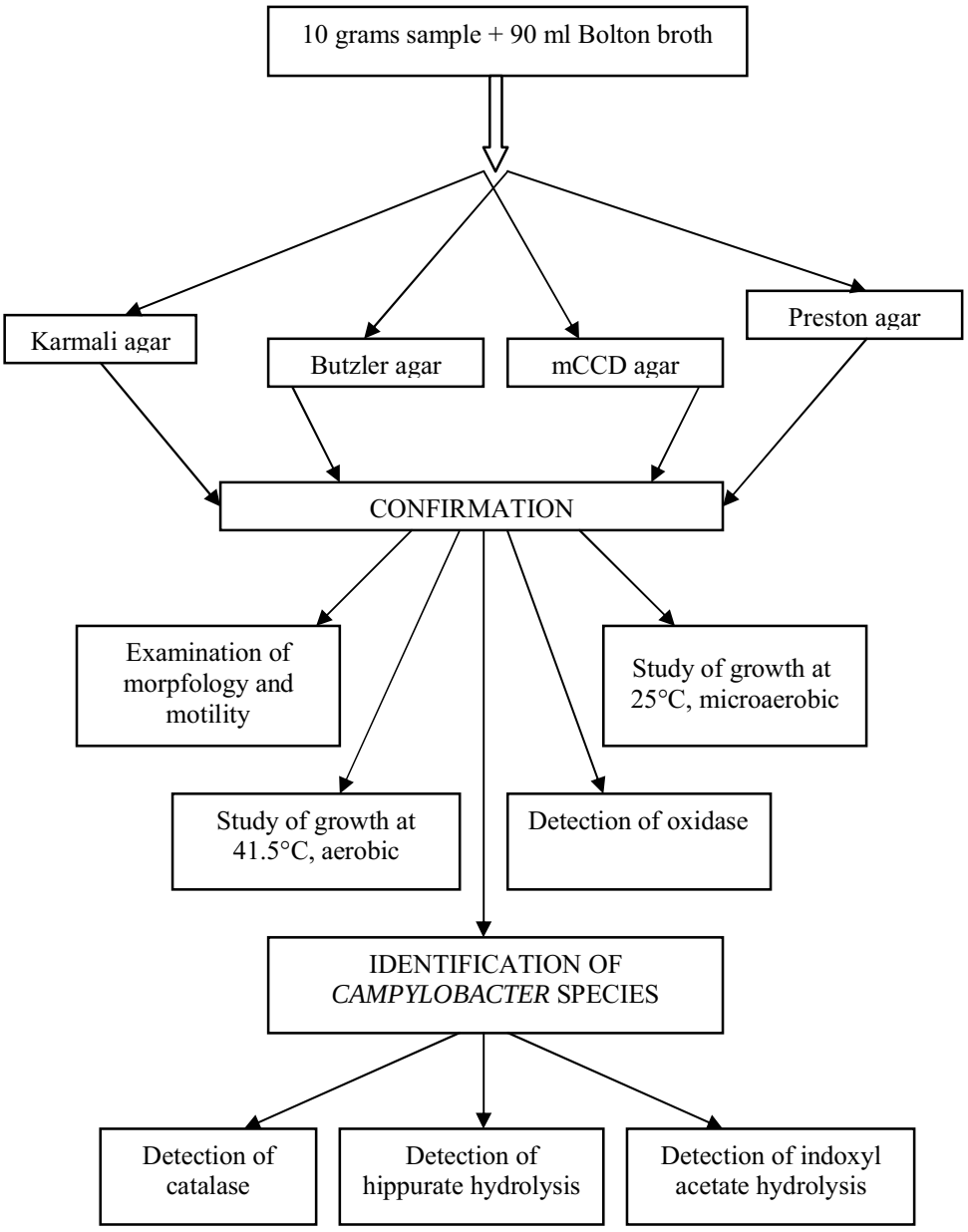


**Fig.1. Confirmation and identification of *Campylobacter spp.***

The identification of *Campylobacter* species was done using ISO 10272-1/2006 standard. Thus, the *Campylobacter* strains were tested on detection of catalase, hippurate hydrolysis and indoxyl acetate hydrolysis. The characteristics of *Campylobacter* species are presented in table 2.

***Table 2***

**Characteristics of *Campylobacter* species**

| Characteristic | *C. jejuni* | *C. coli* | *C. lari* | *C. upsaliensis* |
|---|---|---|---|---|
| Catalase | + | + | + | - or slight |
| Hydrolysis of hippurate | + | - | - | - |
| Hydrolysis of indoxyl acetate | + | + | - | + |

## 2. RESULTS AND DISCUSSIONS

*Campylobacter spp.* was detected in 67 of the 78 analyzed samples, regardless the used selective medium. However, the selective medium has influenced the number of *Campylobacter spp.*

In table 3 are presented the means of results obtained after colony counting and after colony confirmation as *Campylobacter*; there are presented also the percents of confirmation colonies from the number of typical and confirmed colonies.

Although on Butzler media the most typical colonies have growth, after confirmation, on this media was recorded the lowest number of *Campylobacter* colonies. This situation can be explained by a low capacity of this medium to inhibit the competing flora presented in the samples.

***Table 3***

**The means log10 of typical and confirmed colonies of *Campylobacter spp*. and the percentage of confirmed colonies**

| | Plating media (agars) | | | |
|---|---|---|---|---|
| | **Karmali** | **Butzler** | **mCCDA** | **Preston** |
| Mean log10 of typical colonies/g of *Campylobacter spp*.(n = 67) | 3.58 | 3.59 | 3.57 | 3.55 |
| Mean log10 of confirmed colonies/g of *Campylobacter spp*.(n = 67) | 3.48 | 3.46 | 3.53 | 3.50 |
| The percentage of confirmed colonies | 97.2 % | 96.3 % | 98.8 % | 98.5 % |

Following the results, it can be noticed that mCCD and Preston agars combine the best the ability to allow *Campylobacter* growth with the inhibition of competitive bacteria. It was decided to compare mCCD agar with Preston agar because the latter has been shown to give recovery rates of *Campylobacter* strains equal or superior to other selective *Campylobacter* agars. In most laboratories, direct plating onto a selective agar with incubation in a micro-aerobic atmosphere al 41.5 °C for 42-48 hours is the routine technique for the isolation of *C. jejuni* and *C. coli*. Under these standard conditions, mCCD agar performed as well as Preston agar.

The mean log10 cfu/g of *Campylobacter* on Karmali medium was appropriate to mean log10 cfu/g of *Campylobacter* on Preston agar, although initially the mean log10 cfu/g of counted colonies showed a higher number.

After the identification of *Campylobacter* species, there were isolated 49 *C. jejuni* strains and 22 *C. coli* strains; 4 samples contained both strains. The identification of the strains was not influenced by selective media.

## 3. CONCLUSIONS

3.1. mCCD agar and Preston agar have the best selectivity of the number of confirmed positive colonies from the number of counted colonies, offering the best balance between the development of *Campylobacter* germs and the inhibition of competitor germs.

3.2. The selective solid media do not influence the identification of *Campylobacter* species.

3.3. *Campylobacter* numerations are dependent on the used medium, especially for Butzler agar, which has a lower selectivity than mCCD and Preston agars.

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# SEROTYPING SCHEME FOR *CAMPYLOBACTER JEJUNI* BASED ON DIRECT AGGLUTINATION OF HEAT-STABLE ANTIGENS

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**Key words**: *Campylobacter*, serotyping, passive haemagglutination.

## SUMMARY

In many developed countries, cases of enteritis caused by *Campylobacter* are common, *C. jejuni* being detected at a high rate in cases of sporadic diarrhea when compared to rates of other intestinal pathogenic bacilli.
In the serotyping of *Campylobacter jejuni*, two systems of heat-stable and heat-labile antigen are established. The *Campylobacter* antisera are utilized for heat-stable antigen system by passive hemaglutination (PHA) method. Heat-stable specific antigen of *Campylobacter jejuni* extracted by nitric acid is sensitized to the blood cells. When the sensitized cells are mixed with the antiserum, specific reaction occurs and agglutination is observed.
Altogether, 8 serogroups were found in the present study as follows: B (18 %), A (12 %), K (9 %), Y (7,4 %), N (4,4 %), I (4,4 %), J (3 %), D (3 %), respectively. Our study showed that serotypes B and A were the most common types found in analyzed samples. A large number of strains have responded to more than one antiserum which made them to be classified as nontypeable. The proportion of nontypable (38,8 %) isolates is unsatisfactorily and for the serotyping these strains is necessary to use genotypic methods.
*Campylobacter* typing is important for developing strategies to control organisms within the food chain and to establish the sources and routes of transmission of human infection.

In many developed countries, cases of enteritis caused by *Campylobacter* are common, *C. jejuni* being detected at a high rate in cases of sporadic diarrhea when compared to rates of other intestinal pathogenic bacilli. Campylobacters have been isolated from a diverse range of domestic and wild animals and birds. Poultry is now established as a common source of human infection with *C. jejuni*, though numerous other vehicles have been noted. Poultry are colonized with many serotypes that cause the disease in humans.

Two serotyping schemes were developed for the epidemiological characterization of *Campylobacter* isolates. The heat-stable (HS) serotyping scheme of Penner and the heat-labile (HL) serotyping scheme of Lior provided the first methods to subtype *C. jejuni* (Penner and Hennessy, 1980). The application of these techniques to *C. jejuni* isolated from farm animals has proven useful in the investigation of outbreaks and identification of potential reservoirs for human infection (Woodward and Rodgers, 2002).

The purpose of this study is to clarify the epidemiological diversity of *C. jejuni* isolated from broiler carcasses. It is well documented that *Campylobacter spp.* is the predominant cause of human foodborne illnesses in all parts of the world (Park, 2002). Discrimination of *Campylobacter* strains is an important approach to know the contamination route from the standpoint of epidemiology. Some epidemiological links may contribute to the better management or elimination programs in order to avoid illnesses poses to human health as well as proper management of food producing animals for better human health. The Penner's scheme was used as a method of choice for serotyping of *C. jejuni* to discriminate the microorganism isolated from broiler.

## 1. MATERIALS AND METHODS

*Camylobacter spp*. was isolated from chicken carcasses from several slaughterhouses, in different regions of Romania. Only *Campylobacter jejuni* was subjected to serogrouping (Penner and Hennessy, 1980). The identification of *C. jejuni* was performed by biochemical tests. There were studied 67 *Campylobacter jejuni* strains.

In this study, Penner serotyping scheme, using heat-stable antigens (passive haemagglutination tests, PHA), was involved for subspecies identification of *Campylobacter jejuni*. Commercial antisera, haemocytes and solution for the extraction of antigens were used for the tests. Several of these antisera included antibodies to multiply serotypes, as described by Penner and Hennessy (Table 1).

The procedure for obtaining the sensitized cells included the preparation of antigen suspension, the preparation of fixed chick red blood cells (RBCs) suspension. Serotype specific heat stable antigens of *Campylobacter* were extracted by nitrite and absorbed onto fixed chick RBCs. The sensitized cells were agglutinated with homologous antiserum. The commercial kit contains 25 vials with antiserums, which are presented in table 1.

One drop from each antiserum was put on a microplate well over which it was added one drop of the sensitized cells obtained before. In one well place it was put a drop of the control serum as a control for spontaneous agglutination. The interpretation of agglutination was performed after 60 minutes and it is based on general PHA interpretation criteria as presented in table 2.

***Table 1***

**The serogroups and the serotypes for each group**

| | | |
|---|---|---|
| Group A : 1, 44 | Group K : 12 | Group Y : 37 |
| Group B : 2 | Group L : 15 | Group Z : 38 |
| Group C : 3 | Group N : 18 | Group $Z_2$ : 41 |
| Group D : 4, 13, 16, 43, 50 | Group O : 19 | Group $Z_4$ : 45 |
| Group E : 5 | Group P : 21 | Group $Z_5$ : 52 |
| Group F : 6, 7 | Group R : 23, 36, 53 | Group $Z_6$ : 55 |
| Group G : 8 | Group S : 27 | Group $Z_7$ : 57 |
| Group I : 10 | Group U : 31 | |
| Group J : 11 | Group V : 32 | |

***Table 2***

**The interpretation of agglutination**

| Agglutination | Determination | Interpretation |
|---|---|---|
| Agglutination of cells in the center of well | - | Negative reaction |
| Marked agglutination but not all over the bottom of well | + | Positive reaction, the serotype of the tested organism can be determined |
| Agglutination of inconsequential amount of cells in the center | ++ | |
| Uniform agglutination of cells all over the bottom of well | +++ | |

Some *Campylobacter* strains may react with more than one antiserum. These strains are called nontypeable.

## 2. RESULTS AND DISCUSSIONS

Altogether, 8 serogroups were found in the present study, as follows: B (17.91 %), A (11.94 %), K (8.96 %), Y (7.46 %), N (4.48 %), I (4.48 %), J (2.99 %), D (2.99 %), respectively. The results of the study are presented in table 3. Our study showed that serotypes B and A were the most common types found in analyzed samples. A large number of strains have responded to more than one antiserum, which made them to be classified as nontypeable. The proportion of nontypable isolates (38.80 %) is unsatisfactorily and for serotyping these strains it is necessary to use genotypic methods.

*Table 3*

**The number and percentage of *Campylobacter jejuni* isolated serogroups**

| Serogroup | The number of *Campylobacter* strains | The percentage of *Campylobacter* strains |
|---|---|---|
| B | 12 | 17.91 % |
| A | 8 | 11.94 % |
| K | 6 | 8.96 % |
| Y | 5 | 7.46 % |
| N | 3 | 4.48 % |
| I | 3 | 4.48 % |
| J | 2 | 2.99 % |
| D | 2 | 2.99 % |
| Nontypeable strains | 26 | 38.80 % |

The distribution of multiple serotypes in different regions was not surprising considering the mode of *Campylobacter* transmission, via the fecal-oral route from numerous animals, especially poultry, or vehicles contaminated with animal waste (Pettersen *et al.*, 2001).

A previous report from Japan (Iannello, 2004, Koga *et al.*, 2001) described that serotype O was frequently isolated from Guillain-Barre syndrome patients. This serotype was not found in chicken carcasses in our study.

Further studies are needed in order to investigate the serotypes distribution in humans, to establish the importance of poultry as a *Campylobacter* source for human infections and to clarify the epidemiology of *Campylobacter jejuni* infection.

Although serotyping is a practical and valid phenotypic method for epidemiologic typing of *Campylobacter* and has been useful in outbreak investigations, it can produce ambiguous results. This can be due to the occurrence of nontypeable strains, transient antigen expression, and cross-reactivity between certain antigens. The method requires a panel of antisera that is costly to maintain; all these factors limit the use of this technique in surveillance studies.

## 3. CONCLUSIONS

3.1. In this study, there were isolated multiple *Campylobacter jejuni* serotypes from different regions in Romania, fact explained by *Campylobacter*'s mode of transmission.

3.2. Many isolated strains were classified as nontypeable; for the serotyping of these strains there are necessary complementary methods.

3.3. High costs and ambiguous results do not recommend this method in *C. jejuni* surveillance studies.

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# *IN VITRO* EFFICACY OF GENTAMICIN AND *MELLISA OFFICINALIS* ESSENTIAL OIL AGAINST *PSEUDOMONAS AERUGINOSA* ISOLATED FROM DOGS WITH OTITIS EXTERNA

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**Key words:** gentamicin, Mellisa officinalis, Pseudomonas aeruginosa, dog.

**SUMMARY**

Previous investigations regarding the *in vitro* susceptibility of *Pseudomonas aeruginosa* isolated from dogs with otitis externa have pointed out elevated levels of resistance towards several antibiotics, including those contained in most commercial ear medications. Gentamicin is currently mentioned in several therapeutic protocols used in case of canine otitis externa, usually as a topical antibacterial agent. This study evaluated the *in vitro* efficacy of gentamicin alone and combined with *Mellisa officinalis* L. essential oils against canine *Pseudomonas aeruginosa* strains. The antipseudomonal synergistic interaction between the two products was measured by a broth microdilution method in accordance with CLSI protocols and analyzed based on the values of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), fractional inhibitory concentration (FIC) and fractional bactericidal concentration (FBC) indices. 70% of the bacterial strains were more susceptible to this combination compared to the antimicrobial alone. The inhibition of bacterial growth in case of some gentamicin resistant strains was observed with the individual extract and also when the herbal extract was used in lower concentrations with ineffective antibiotics. Further *in vitro* and *in vivo* studies are needed in order to establish a therapeutic recommendation.

Otitis externa in dogs is usually described as a pattern of cutaneous disease that possess a complex pathogeny including predisposing factors (pendulous pinnae, stenosis, neoplasms, hair in the ears, excessive cerumen production, trauma, and high humidity), primary causes (parasites, hypersensitivity, keratinization disorders, foreign bodies, ear gland disorders, and autoimmune diseases) and perpetuating factors (bacteria, yeasts, pathologic changes, such as glandular hyperplasia, epithelial folds, neoplasia, edema, mineralization, and fibrosis). An effective management of otitis externa in dogs should consider all these conditions and causes and one particulary important aspect reffers to controlling the bacterial organisms responsible for the infection. Several authors mentioned *Pseudomonas aeruginosa* as a bacteria commonly involved in canine ear infections and also the multidrug-resistant profiles

for these isolates (Petersen et al., 2002; Moulari et al., 2007; Schick et al., 2007). Previous investigations regarding the *in vitro* susceptibility of *Pseudomonas aeruginosa* isolated from dogs with otitis externa have pointed out elevated levels of resistance towards several antibiotics, including those contained in most commercial ear medications (Hariharan et al., 2006; Lyskova et al., 2007; Zamankhan et al., 2010). Gentamicin is indicated by several therapeutic protocols used in case of canine otitis externa, usually as a topical antibacterial agent (Petersen et al., 2002; Lyskova et al., 2007; Zamankhan et al., 2010).

Nowadays, a multitude ofbioactive herbal extractions or constituents are investigated as intermediates for use in the development of chemotherapeutic agents that may be applied in the management or treatment of infections, especially for those determined by multiresistant bacteria, such as *Pseudomonas aeruginosa*. The aromatic herb *Melissa officinalis* L. has many medical attributes, acting as an antibacterial, antiviral, antispasmodic, aromatherapeutic, digestive soother, emmenagogue, anti-pyretic, antioxidant, immuno-stimulant, antihormonal, anti-cancer (Mimica-Dukic et al., 2004; Canadanović-Brunet et al., 2008; Hăncianu et al., 2008; Niculae et al., 2009; Stanojevic et al., 2010).

The aim of the present study was to investigate the *in vitro* antibacterial efficacy of gentamicin alone and combined with *Mellisa officinalis* L. essential oils against canine *Pseudomonas aeruginosa* strains.

## 1.MATERIALS AND METHODS

The set of microorganisms elected for this assay included *Pseudomonas aeruginosa* strains (n=10) isolated from clinical cases of canine otitis externa and a standard strain, *Pseudomonas aeruginosa* ATCC 27853. These isolates, identified by standard microbiological methods, were also screened for their susceptibility towards antimicrobial agents, displaying different levels of antibiotic resistance, resistant to ampicillin (90%), amoxicillin (80%), cephalexin (70%), colistin (100%), neomycin (80%), gentamicin (80%), tetracycline (100%), oxytetracycline (100%), streptomycin (100%) and sensitive to enrofloxacin (70%), cefquinome (60%) and florfenicol (100%). In order to perform the antimicrobial screening, the bacterial isolates were cultured overnight at 37◦C on Brain Heart agar (Oxoid). Colonies collected from each twenty-four hours bacterial culture were diluted in sterile saline and the optical density was adjusted according to the tube

0.5 of McFarland' scale to prepare a standardized inoculum (1.5 x $10^8$cfu/ml).

Minimal inhibitory (MIC) and bactericidal (MBC) concentrations were determined by a broth microdilution method in accordance with CLSI protocols: series of twofold dilutions of each essential oil and ethanolic extracts, ranging from 4% to 0.125% (v/v), were mixed with an equal volume of bacterial suspension and incubated for 24h at 37°C. The MIC was the lowest concentration of herbal extractions that inhibited the visible growth (no turbidity), when compared to the control. Afterwards, 10µL of each well were transferred to Mueller Hinton agar plates and incubated for 24h, at 37°C. The lowest concentration associated with no visible growth of bacteria on the agar plates was considered the MBC.

Fractional inhibitory concentration (FIC) and fractional bactericidal concentration (FBC) indices for gentamicin and *Mellisa officinalis* L. essential oils were determined by checkerboard method according to Stanojevic´ et al. (2010). FIC index was calculated by FIC (MIC of drug A in combination with drug B/MIC of drug B alone). FIC of <0.5 was defined as synergy, an FIC index of > 0.5 to 1 was defined as additive or indifferent, and FIC of > 4.0 was defined as antagonism.

## 2. RESULTS AND DISCUSSION

***Table1***

**The antimicrobial activity of *Mellisa officinalis* L. essential oils alone and tested in combination wtih gentamicin against *Pseudomonas aeruginosa* strains**

| *Pseudomonas aeruginosa* strains | *Mellisa officinalis* L. % (v/v) | | | *Mellisa officinalis* L. + gentamicin % (v/v) | | | FIC |
|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC/MBC | MIC | MBC | MIC/MBC | |
| S1 | 2 | 2 | 1 | 1 | 2 | 0.5 | < 0.5 |
| S2 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | <0.5 |
| S3 | 1 | 1 | 1 | | 0.5 | 1 | <0.5 |
| S4 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | <0.5 |
| S5 | 4 | >4 | - | 4 | 4 | 1 | - |
| S6 | >4 | >4 | - | >4 | >4 | - | - |
| S7 | 2 | 2 | 1 | 2 | 2 | 1 | <0.5 |
| S8 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | <0.5 |
| S9 | >4 | >4 | - | 4 | >4 | - | - |
| S10 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | <0.5 |
| *Ps. aeruginosa* ATCC 27853 | 1 | 1 | 1 | 0.5 | 1 | 0.5 | <0.5 |
| *Combination* | *FIC* | | | *FICI* | | | |
| *Mellisa officinalis* + gentamicin | 0.5<br>0.125 | | | 0.625 | | | |

The minimum inhibitory (MICs) and bactericidal (MBCs) concentrations obtained by the microdilution protocol were very similar (table 1), suggesting that the bacteriostatic and bactericidal activity are displayed at similar concentrations.

*Melissa officinalis* essential oils expressed promising antibacterial properties against tested *Pseudomonas aeruginosa* strains also by enhancing the activity of gentamicin (table 1). 70% of the bacterial strains were more susceptible to this combination compared to the antimicrobial alone. The inhibition of bacterial growth in case of some gentamicin resistant strains was observed with the individual extract and also when the herbal extract was used in lower concentrations with ineffective antibiotics.

Management of chronic recurrent otitis externa requires diagnosis and control of the predisposing factors, primary causes, and perpetuating factors. Topical and systemic antimicrobials represent part of the therapy protocols and due to the antimicrobial resistance elevated level observed for prevalent bacteria in canine otitis externa we may observe the chronic and / or recidivant course of this pathology.

Plant essential oils are a potentially useful source of antimicrobial compounds. Previous researches have suggested that essential oils obtained from *Melissa officinalis* L. display important *in vitro* inhibitory effects on the bacterial growth in case of human and animal originating isolates (Mimica-Dukic et al., 2004; Moulari et al., 2007; Cheryl et al., 2008; Niculae et al., 2009; Stanojevic et al., 2010). The lemon balm oil sample tested by Hancianu et col. (2008) exhibited antibacterial activity only against Gram-positive strains.

It is often quite difficult to compare the results obtained from these different studies, because the compositions of the essential oils can vary greatly depending upon the geographical region, the variety, the age of the plant, the method of drying and the method of extraction of the oil.

## 3. CONCLUSIONS

3.1. Data from our results point out the antibacterial potential of *Melissa officinalis* essential oils against multidrug resistant bacteria.

3.2. More studies (*in vivo* efficacy, *in vitro* toxicity assays) need to be conducted in order to consider and to validate its use as a phytotherapeutic product in canine otitis externa.

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# ERYTHEMA MULTIFORME IN DOGS – A REVIEW

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**Key words:** erythema multiforme, dog.

**SUMMARY**

Erythema multiforme (EM), an uncommon feature in small animal practice, is described as an immune-mediated dermatological syndrome expressed by an acute, dramatic erythematous skin eruption (erythematous macules, erosions, and ulcerations) with life-threatening potential. In fact, most of the case reports refer to dead animals or to animals subjected to euthanasia. The complex pathogenesis is not fully understood, but in dogs it appears to be linked most frequently to drugs administrations, infections, or internal disorders. It posses a distinctive histopathology-characteristic lymphocytic interface dermatitis with hydropic degeneration of basal cells and keratinocyte apoptosis, indicating a cell-mediated hypersensitivity reaction, leading to epidermal and follicular wall irreversible damages. Therapeutic protocols suggesting the elimination of the triggering factors and the suppression of the immune response have little efficacy and the usefulness of some medications (e.g glucocorticoids) in canine erythema multiforme is still controversial.

Erythema multiforme (EM) is an uncommon feature in small animal practice, more often seen in dogs compared to cats. No age or sex predilections have been mentioned in dogs and cats, but regarding the breed several retrospective studies had indicated that German Shepherd, Pembroke Welsh Corgis, Old English Sheepdogs, Chow Chows, Cairn Terriers, Bearded Collies, Bobtail, Dobermann pinscher presented a statistically significant increased risk of developing EM (Scott and Miller, 1999; ).

As for the clinical aspects associated with erythema multiforme, there are descriptions of an acute, dramatic erythematous skin eruption (erythematous macules, erosions, and ulcerations) with life-threatening potential. Most cases refer to animals showing skin erythema around the ears, head and neck, and soon on whole body, and with lesions with a typical pattern – annular or arciform, erythematous macula, papules and plaques that become enlarged starting from the centre, spread centrifugally and leave a central clear area (“target” lesion); these “target” lesions tend to become vesicular or bullous and also necrotic, resulting ulcers complicated by secondary bacterial infections. This typical aspect is usually observed on body sites including the ventrum

(especially axillae and groin), mucocutaneous junctions, oral cavity (tongue, oral mucous membrane), pinnae, footpads and central dorsal regions (McMurdy, 1990; Hinn *et al.*, 1998).

Majority of reported clinical cases present fever, severe depression and dehydration ensued (8% dehydrated), and extreme pain and because usually the therapy proves to be unsuccessful, these animals were euthanasied.

As for the conditions responsible for this pathological state, the general opinion is that the erythema multiforme pathogenesis is not completely understood, but it is attributed to a host-specific cell-mediated hypersensitivity response with dramatic consequences on skin cells (epidermal and follicular wall keratinocyte apoptosis). It is postulated that T-helper cells (CD4+), stimulated by antigen-presenting Langerhans' cells, become sensitized against keratinocytes and cause damage to the keratinocytes by releasing lymphokines, such as interferon-alpha and tumor necrosis factor-beta. Cytotoxic T-cells (CD8+) may also play a role in keratinocyte damage (Favrot *et al.*, 2000; Scott *et al.*, 2001; Gross *et al.*, 2005).

Mild forms are considered idiopathic or linked to viral and bacterial infections (especially canine distemper, canine parvovirus 2), while the severe forms are associated with drugs reactions (Favrot *et al.*, 2000).

Regarding the therapies that led to EM in dogs, several substances are mentioned: antimicrobials (enrofloxacin, penicillin, amoxicillin, amoxicillin – clavulanic acid, cephalexin, lincomycin, chloramphenicol, trimetoprim-sulphamethozaxol (Delmage and Payne-Johnson, 1991), gentamicin, tetacycline), insecticids (chlorpyrifos, organophorphores) (Noli *et al.*, 1995; Rosenbaum and Kerlin, 1995), antihelmintics (ivermectine, levamisole, diethylcarbamazine), antifungics (5-fluorocytosine), antiinflamatoires (aurothioglucose), L-thyroxine, phenobarbital (Hinn *et al.*, 1998).

McMurdy described a case of severe erythema multiforme in a dog which had received many medications for recurring dermatosis. The disease was characterized by crusting of the lips and eyelids; ulceration of the oral mucosa; vesiculation and ulceration of axillary, inguinal and perianal skin, between footpads, and on the inside surfaces of the pinnas; keratoconjunctivitis sicca; and bilateral corneal ulcers. These ulcers healed slowly, but tear production did not resume after resolution of the skin lesions. Cutaneous lesions were characterized histologically by necrosis of individual cells of the epidermis and intrafollicular epithelium as well as focal areas of full-thickness epidermal necrosis and

superficial perivascular lymphohistiocytic infiltration. The aetiology was thought to be a reaction to sulfonamide administration.

Differential diagnoses include several severe dermatological pathologies: superficial and deep bacterial infection, superficial and deep fungal infections, demodectic mange, pemphigus foliaceus and pemphigus vulgaris, bullous pemphigoid, systemic lupus erythematosus, epitheliotropic lymphoma, thermal or chemical burns (Scott *et al.*, 2001; Gross *et al.*, 2005).

Confirmatory diagnosis is based on the history, clinical and histopathological findings (cutaneous and mucocutaneous biopsy specimens with aspects of lymphocytic interface dermatitis with hydropic degeneration of basal cells and keratinocyte apoptosis). For the mild forms or in case in which the causative drug can not be clearly determined, laboratory tests suitable for the confirmation of a possible viral or bacterial etiology are recommended (e.g., molecular methods to detect epitheliotropic viruses pathogenic in dogs, such as: distemper, papilloma-viruses, parvoviruses, and herpesviruses) (Hinn *et al.*, 1998; Paitaki *et al.*, 2001).

The therapy or the management of EM can prove to be challenging due to the lack of information about this pathological state.

The main objectives are the elimination of the triggering factors (if this cause can be identified) and the institution of long-term supportive measures (intravenous fluid therapy to combat dehydration and the shock, analgesia). Several authors reported that the removal of the associated trigger factor and supportive care resulted in resolution of the erythema multiforme within 1–2 weeks. Some dogs with severe idiopathic erythema multiforme were successfully managed with glucocorticoids, azathioprine, pentoxifylline (5 to 10 mg/kg 3 times a day) or Atopica. Azathioprine, sulfasalazine and cyclosporine have been used as palliative treatments in resistant cases.

Their usefulness in canine erythema multiforme is controversial. Itoh *et al.* (2006) reported a case of EM (erythematous skin lesions at the axillae, groin, mucocutaneous junctions, and pinnae) in a 5-year-old female border collie that responded to a therapy that included azathioprine, prednisolone, and a hypoallergenic diet.

Also, antiinflammatory doses of prednisolone (0.5 to 1.0 mg/kg/day) may be given to decrease the inflammation and pruritus when present but are controversial because they can also exarcebate some cases

The antimicrobial treatment is also questioned as some of the antimicrobial agents currently used in order to prevent sepsis and local

secondary infections may act as triggering factors as suggested by literature.

The presented information suggests the importance of EM in the differential diagnosis of small animals dermatologic disease, due to unclear pathogenesis, limited treatment options and life-threatening potential.

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# EPIDEMIOLOGICAL SURVEILLANCE OF WILD ANIMALS POPULATIONS IN NORTH MOLDAVIA IN 2006-2008

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**Key words**: wild animals, profilaxy, diagnosis

**SUMMARY**

In 2006-2008, in 4 counties from north Moldavia (Bacău, Botoşani, Iaşi, Suceava) with 227 hunting areas, we established the dynamics of the populations of several wild species: bears (*Ursus arctos*), deers *(Cervus elaphus)*, fallow deers *(Dama dama)*, roe deers (*Capreolus capreolus*), wild boars *(Sus scrofa)*,wolves (*Canis lupus*), rabbits (*Lepus europaeus*).
County laboratories of the DSVSA in the area offered us the information concerning the status of diagnosis and prevention measures implemented for these particular species in the same time frame.
General conclusions: 67 % of the shot or transferred species of wild animals were the subject of laboratory exams; vaccinations are performed with an important approximation rate.

In the circumstances of a more and more obvious anthropisation of natural environments, careful monitoring of the evolution of wild animals populations and of their health status is compulsory, in order to mantain the health status of domestic animals, the contact between them being more and more frequent. We must not neglect the importance of wild fauna conservation, seing that Romania inhabits even species that have gone extinct in other european countries. The diagnostic, prophylaxy and prevention of major animal disease is a compulsory condition seeing the present interferations between the natural and the anthropic.

## 1. MATERIAL AND METHOD

The species investigated in this study are the bear (*Ursus arctos*), the deer (*Cervus elaphus*), the fallow deer (*Dama dama*), the roe-deer

(*Capreolus capreolus*), the boar (*Sus scrofa*), the wolf (*Canis lupus*) and the hare (*Lepus europaeus*).

We studied 4 counties in north Moldavia: Bacău, Botoşani, Iaşi and Suceava, and for each of them, we centralised the data obtained from the managers of the hunting areas concerning the livestock of the mentioned species for 2006-2008, the optimum livestock taking into account the characteristics of the biotopes and the dinamics of the populations for the established period of time.

Through a tight collaboration with the Sanitary Veterinary Directions and for Food Safety, we obtained information concerning the veterinary prophylactic and diagnosis actions mentioned in the Program of Surveillance, Prevention, Control and erradication of disease in animals, of animal – human transmissible disease, animal protection, and environment protection.

## 2. RESULTS AND DISCUSSIONS

The managers of the hunting areas (County associations of hunters and fishermen and Silvuc Directions) in the counties included in this study gave us the following data concerning the wildlife species taken in study, data centralised in Table 1 and Table 2.

***Table 1***

**Livestocks of bear, deer, fallow deer and roe deer for the counties and the timeframe studied**

| COUNTY | BEAR | | | DEER | | | FALLOW DEER | | | ROE DEER | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| BC | 196 | 196 | 197 | 674 | 1053<br>331♂<br>722♀T | 1039<br>333♂<br>706♀T | - | - | - | 4970 | 4911<br>1577♂<br>3334♀T | 5041<br>1588♂<br>3453♀T |
| IS | - | - | - | 290 | 284 | 309 | - | - | - | 3661 | 3731 | 3851 |
| SV | 283 | 275 | 275 | 4125 | 4188 | 4252 | 80 | 75 | 60 | 5046 | 5173 | 5261 |
| BT | - | - | - | 242 | 173 | 168 | - | - | - | 2506 | 2784 | 2929 |

***Tabel 2***

**Livestocks of hare, boar and wolf for the counties and the timeframe studied**

| COUNTY | HARE | | | BOAR | | | WOLF | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| BC | 27368 | 25257 | 26141 | 1334 | 1386 | 1505 | 117 | 118 | 118 |
| IS | 49215 | 49740 | 50460 | 816 | 840 | 865 | - | - | - |
| SV | 28765 | 28420 | 29580 | 2548 | 2616 | 2276 | 220 | 248 | 236 |
| BT | 43736 | 39910 | 39610 | 574 | 687 | 697 | - | - | - |

The information we were offered made it possible to follow the dinamics of the wildlife populations, the increase or decrease of livestock according to the values of hunting plans, with the general tendency of decrease of habitats and last but not least, with the financial resources alocated differentially to different hunting areas. (Fig. 1-8).

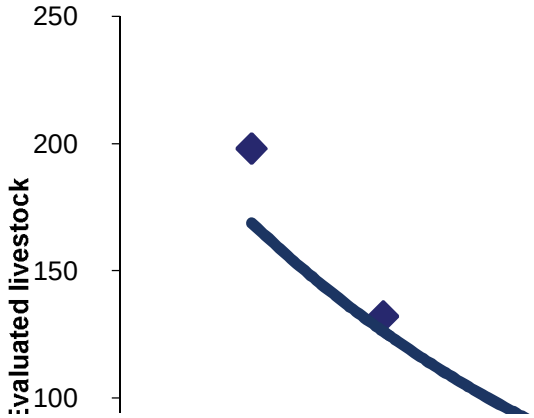


**Fig. 1. Dinamics of deer livestock in Botosani according to hunting areas managers**

Deer livestocks show a significant decrease for the areas managed by Silvic directions, being much underoptimal. In the case of hunting areas managed by the AJVPS we can notice the maintenance of the livestock at a constant value, higher than the optimum livestock.

The cause of this phenomenon can be the retrocedation of forests, that drastically diminished the areal.

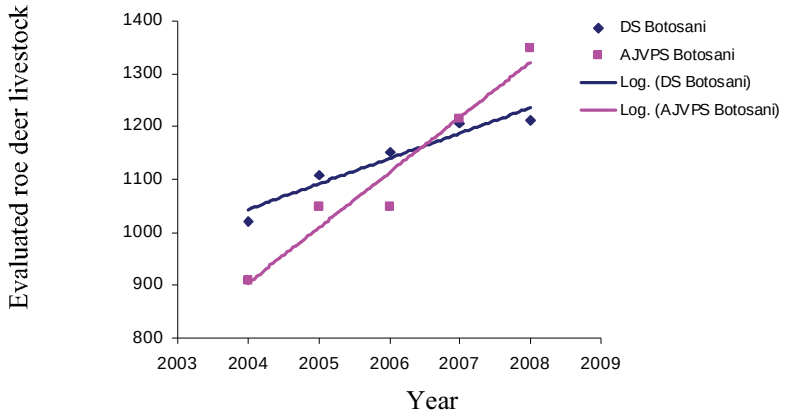


**Fig. 2. Dinamics of roe deer livestock in Botosani according to hunting areas managers**
**Evaluated roe deer livestock**

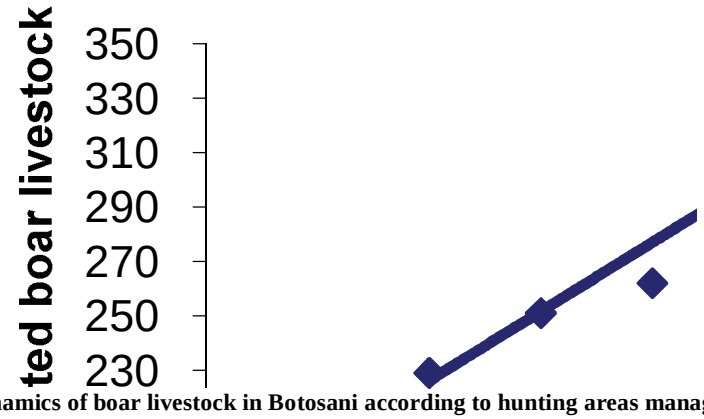


**Fig. 3. Dinamics of boar livestock in Botosani according to hunting areas managers**

Roe deer livestocks show a constant increase in both situations, fact that can be explained through the fragmentation of forests, fact that is favourable to this species, that preferrs small forests alterning witl open fields.

Boar livestocks are well represented and increasing, fact explained by their access to agricole cultures appeared after deforresting and by the adaptability of the species.

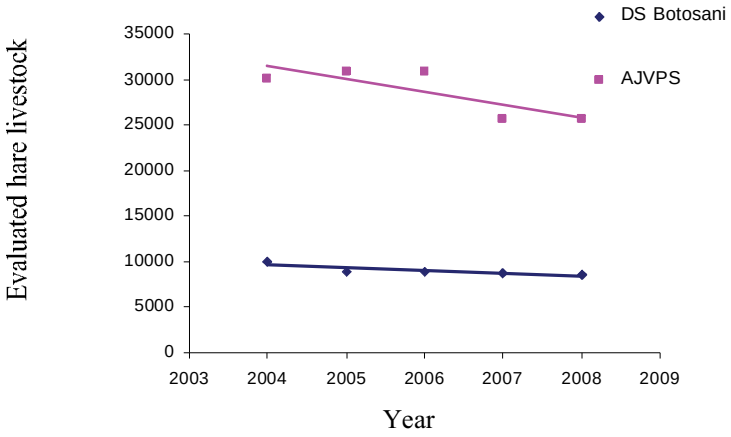


**Fig. 4. Dinamics of hare livestock in Botosani according to hunting areas managers**

Analyzed hunting areas are favourable to the development of healthy hare populations, but are underoptimal on hunting areas managed by silvicultors. The reason could be the prefference of this species for agricultural areas, mainly managed by hunters associations.

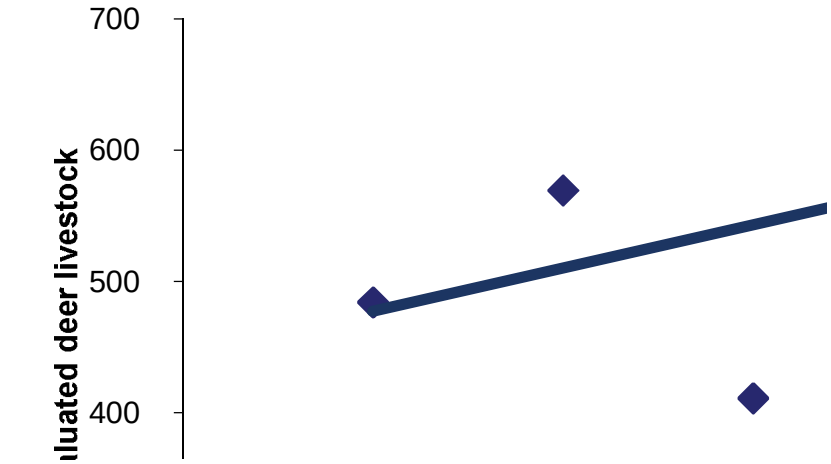


**Fig. 5. Dinamics of deer livestock in Bacau according to hunting areas managers**

Deer livestocks are optimal for both managers, with increasing tendencies.

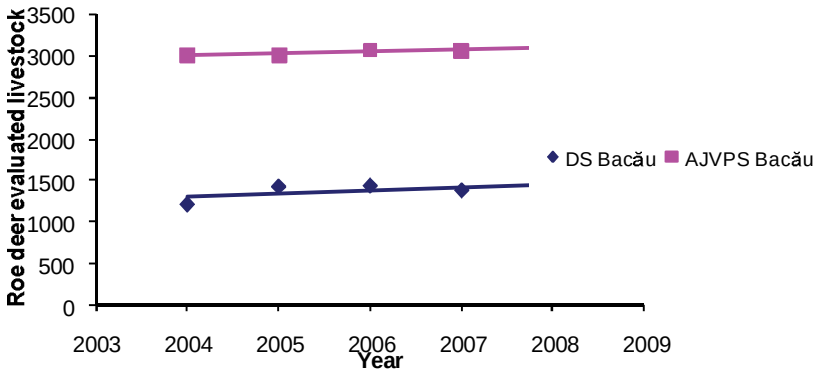


**Fig. 6. Dinamics of roe deer livestock in Bacau according to hunting areas managers**

Roe deer populations are overoptimal and increasing, fact due to the high plasticity of the species. Deforresting favours the species by creating areas with herbal vegetation.

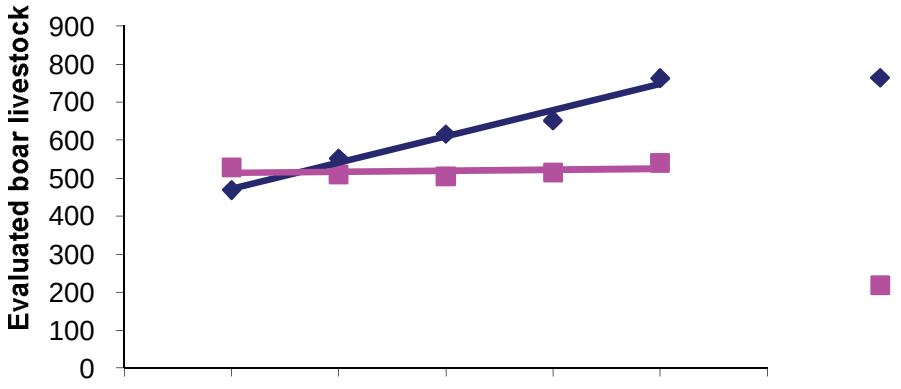


**Fig. 7. Dinamics of boar livestock in Bacau according to hunting areas managers**

By far the most adaptable species is the boar, who develops great in variable conditions. The phenomena was analyzed in several european countries, so hunting became unrestrited for this species.

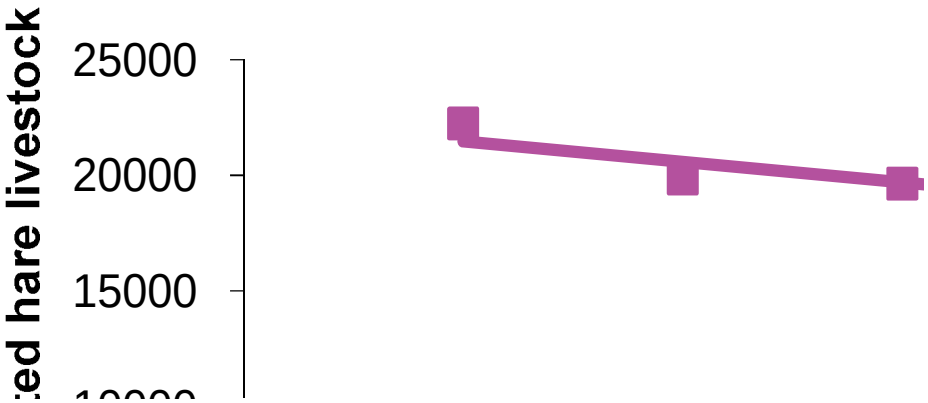


**Fig. 8. Dinamics of hare livestock in Bacau according to hunting areas managers**

Evolution of hare populations corresponds to a good cinegetic managing, livestocks having only normal fluctuations.

Anual monitoring of numeric evolutions of wildlife livestocks offer an overall image of the way hunting areas are managed, of changes concerning the ration prey:predator, fact observed for example after making the wolf a protected species, of the different pathological processes that can affect different species.

The Program of surveillance, prevention, control and erradication of disease in animals mentions a few compulsory exams performed on some wildlife species, such as the trichinelloscopic examination of all

boar and bear carcasses, serological and virusological examinations to identify swine pestis in boars, transmissible encephalopathies in deer, roe deer and fallow deer; livestocks of wild carnivores, especially wolves and foxes are subject of monitoring and vaccination against rabies, hares are monitored for brucellosis in endemic areas.

But there are several pathological processes, transmissible or not, that can harm wildlife populations, either spontaneously or as consequence of more and more frequent contact with domestic animals, given the fact that grazing in forests has become more and more frequent; these diseases are not subject of an official monitoring, but they may severely influence the wildlife, either numerically or as biological value. The studies we performed showed for example that parasitosis are widely spread, sarcocystosis being identified in a very high percent of the exained samples.

Even for the disease of cumpolsory supervision, the number of samples examined are only a very small percentage of the total livestock. The data we centralised for 2006, 2007 and 2008 show less then 1% of positive cases of trichinellosis, but the number of animals examined are is also a very small part of the total livestock reported by managers of hunting areas; given the fact that the bear is a protected species and extraction rates are very small (1-3/year/the four counties) we can say that we do not have an overall image of trichinellosis incidence. In Bacau, in 2007, from a livestock estimated at 196 exemplaries, only one body was examined, that proved negative for trichinellosis; in 2008 a single body was examined as well, but it proved positive. Thus, it is impossible to sketch a pertinent overall image of the incidence of trichinellosis for this species.

For boars, with bigger livestocks and so more generous extraction rates, the results of the trichinelloscopic examination are shown in the next table.

***Table 3***

**Trichinellosis incidence in bears in 2006-2008 in studied areas**

| | Bacău | | | Botoşani | | | Iaşi | | | Suceava | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| Estimated livestock | 196 | 196 | 197 | 0 | 0 | 0 | 0 | 0 | 0 | 283 | 275 | 275 |
| Examined samples | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| Positive samples | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |

For boars, with bigger livestocks and so more generous extraction rates, the results of the trichinelloscopic examination are shown in the next table.

***Table 4***

**Trichinellosis incidence in boars in 2006-2008 in studied areas**

| | Bacău | | | Botoşani | | | Iaşi | | | Suceava | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| Estimated livestock | 1334 | 1386 | 1505 | 574 | 687 | 697 | 816 | 840 | 865 | 2548 | 2616 | 2276 |
| Examined samples | 218 | 288 | 141 | 79 | 112 | 185 | 79 | 112 | 185 | 122 | 201 | 308 |
| Positive samples | 2 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 3 | 4 |

One can easily notice that the number of examined samples is a small percent of the total livestock reported; it is the same case for transmissible encephalopathies of cervides.

***Tabel 5***

**Incidence of transmissible encephalopathies in cervides in 2006-2008 for the studied areas**

| | Bacău | | | Botoşani | | | Iaşi | | | Suceava | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| Estimated cervides livestock | 5644 | 5964 | 6080 | 2748 | 2957 | 3097 | 3951 | 4015 | 4160 | 9251 | 9436 | 9573 |
| Examined samples | 113 | 138 | 124 | 229 | 274 | 206 | 263 | 297 | 301 | 211 | 258 | 22 |
| Positive samples | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

As one can notice, for cervides the number of examined animals is even smaller than the total livestock.

As for classical swine fever, we only received centralised data from Bacau and Botosani (Table 6).

***Tabel 6***

**Incidence of classical swine fever in boars in 2006-2008 in Bacau and Botosani**

| | Bacău | | | Botoşani | | |
|---|---|---|---|---|---|---|
| Year | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| Estimated livestock | 1334 | 1386 | 1505 | 574 | 687 | 697 |
| Examined samples | - | 420 | 368 | 144 | 208 | 246 |
| Positive samples | - | 0 | 0 | 0 | 0 | 0 |

Coroborration of data obtained from the AJVPS, Silvic directions and Veterinary Laboratories prove similar situations for all the disease of the wildlife subject to compulsory diagnosis: a very small percent of the livestock gets to be examined for the pathological states mentioned in

the national programme; infections and parasitic disease that are not mentioned in this program are not monitored, thogh their presence is highly damaging.

## 3. CONCLUSIONS

3.1. Generally, wildlife populations are well represented and normally developped. Exception is the deer in Botosani, where measures of protecting the species against the anthropic pressure are necessary.

3.2. Roe deer and boar have an ascendent numeric evolution due to the plasticity of the two species, but also due to the oportunities involuntarily offered by deforresting.

3.3. One can notice relatively high differences between the evolution of wildlife populations in different managers care. The reason is represented by managing and bonity of different hunting areas.

3.4. The socio economical frame of our country as well asthe decrease level of cinegetic research and education must be urgently reconsidered. Despite the fact that game mortalities have a major quantum, they are not evidentiated in evidence charts. Pathological processes surely have a considerable contribution, which justify the reesearch oriented on diagnosis, surveillance and prevention.

3.5. Compulsory diagnosis measures are insufficient to create an overall image of the health status of wildlife.

3.6. A small percent of the total livestock is being examined for major pathological states.

3.7. The national program of wildlife surveillance doesn’t mention a thing on major disease that bring major prejudice to the health, vitality and biological value of the phauna. Thus, it is important to reconsider the importance of surveillance and maintenance of the health status of the game, the implementation of efficient measures, the education of hhunters in order to identify and signal the animals with evidence of disease or to prelevate bodies found in the forest and show them to abbilitated institutions.

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# MILK CATTLE WELFARE IN ACCORDANCE WITH THE POLLUANT FACTORS IN VALCEA AREA AND THE HAEMATOLOGICAL PROFILE

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**Key words:** animal welfare, milk cattle, haematologic status, polluting factors

**SUMMARY**

The aim of the investigations presented in this paper was to follow the changings made by the polluting factors in the haematological profile and the use of the latter in monitorising the cattle welfare in this area, especially the milk cattle. 40 blood samples taken between September-November 2009 were analyzed. These samples were taken from milk cattle of different ages and physiological states, from areas situated near the industrial platform where productivity problems frequently appear (decrease in the milk production, infertility, repeated reproductions, unjustified weight loss).
The drawing and sending of the samples was made by the official veterinarian doctor from the outskirts of Ramnicu Valcea town. The samples were sent for analysis to the Institute of Diagnosis and Animal Health in Bucharest, in the National Reference Laboratory for Animal Welfare, where an evaluation of the animal welfare is done through a national surveillance programme in the context of protecting man's health and of eco-san-genesys. Changes of the haematological status were noticed translated through the changes of the haematocrit value, the average erythrocyte volume, thrombocyte number, leucocyte formulae and citomorphologic examination. This data will be used to make and implement an integrated programme of animal welfare surveillance and to carry out a feedback by offering the results to the cattle breeders.

Blood, both because of its varied functions and the direct relationship he has with all bodies, reacts sensitive to changes produced in the body by internal factors and/or external. Blood examination gives valuable indications for the discovery not only of blood tissue disease or nutritional status of animal suffering and other organs or the action of external agents (bacteria, viruses, toxic compounds) (Parvu, 1992). Therefore, haematological examination was chosen as a *biomarker* for monitoring the health of cattle from areas with potential polluter in the county of Valcea.

In the county of Valcea, Ramnicu Valcea chemical plant site area (OLTCHIM, USG, CET, Vilmar) is considered a critical area for a long time in terms of air pollution. Although economic growth is the Valcea county, industries have developed from the natural resources in the last half century, and constituted the largest sources of pollution, while

bringing serious environmental damage and animal health and therefore human.

## 1. MATERIALS AND METHODS

The aim of investigations presented in this paper was to track changes induced by pollutants discharged, haematological profile and its use in monitoring the health of cattle in this area, particularly dairy cows. We have analyzed 40 blood samples taken from dairy cows of different ages and physiological states of households in areas near the industrial area and frequently being in productivity problems (low milk production, infertility, monte repeated, unjustified weakening, etc.).

Samples were taken from each batch consisting of 10 animals belonging to each of the studied areas, especially areas with potential polluter, as follows: 1. Rm city adjacent area, considered as the area most affected by pollution are the main sources of chemical pollution, traffic, burning fuel (natural gas, coal, fuel oil, light fuel oil), waste incineration, etc. 2. Surface area of coal mines, natural gas comes Alunu; 3. Oil extraction area Babeni areas and members; 4. Hilly and mountainous areas, regarded as less affected by pollution.

Collecting and submitting samples was carried out by an official veterinarian of the adjacent area of Ramnicu Valcea. Blood samples were taken in the morning before the animals go to pasture, to prevent possible changes in testing during the day. Sampling was done with minimum trauma, otherwise there is a risk of release of tissue factors that can activate the extrinsic coagulation path. Blood was taken from the jugular vein, after a preliminary training site of choice (cutting and disinfecting with alcohol). Venepuncture was not to favor the passage of the farm to the movement of fibrinolytic mediators from vascular endothelial cells but also to prevent hemolysis, which may result in erroneous values, resulting in increasing inter alia (false) haematocrit (Orasanu, 2007).

Blood was collected in anti-coagulant vacutainere ethylene-diamine-tetra-acetic acid (EDTA), making it one sample under vacuum, then it was shaken gently to blend. Vacuum sampling system has some practical advantages: it is quick, easy, and carried in a closed system, providing the security that is taking. He checked the closing of the tubes to avoid evaporation causing hemoconcentrație plasma, the source of errors. Samples were sent for analysis at the Institute for Diagnosis and Animal Health in Bucharest, the National Reference Laboratory for Animal Welfare, where animal welfare assessment is carried out through

a national surveillance program in the context of protection of animal health, human and eco-san-genesys.

Haematological examination was performed in the laboratory of Haematology, using automated hematology analyzer Coulter Counter model Ac T 5 diff. CP.Quantitative hematology included determining the following parameters: erythrocyte count, hemoglobin, haematocrit, erythrocyte-derived indices (MCV, HEM, MCHC), WBC, platelet count (platelets).

Haematological examination included qualitative determination in leukocyte formula and cytomorphological examination and peripheral blood smear was performed with Leica DM LS2 microscope.

## 2. RESULTS AND DISCUSSION

The results (table 1, 2, 3, 4) were analyzed comparing animals reared in the four areas studied, and pointed to notice any changes in the parameters that are part of hematology, considered as a test examination of the forecasting group, and identify changes related to state health specialist for the production of dairy cows. All cows in this area are increased by a similar manner on pasture until late autumn to early spring.

***Table 1***

**Mean quantitative haematological examination in cattle in the GP - Group I Rm.Valcea city - adjacent area**

| Crt. Nr. | RBC mil/mm$^3$ | (HGB) g/dl | (HCT) % | MCV $\mu^3$ | MCH pg | MCHC g/dl | WBC mii/mm$^3$ | PLT mii/mm$^3$ |
|---|---|---|---|---|---|---|---|---|
| **Aver.** | 6.86 | 9.8 | **28.8** | **37.8** | 14.3 | 34.4 | 8.9 | **174.1** |
| **N.V.** | 6.5±1.4 | 10.2±1 | 35±3 | 53.4±2.2 | 16±2.5 | 29.1±2.1 | 8.0±1.5 | 1-8 |

***Table 2***

**Mean quantitative haematological examination in cattle in the GP - Group II Surface coal mining area Alunu – Berbeşti**

| Crt. Nr. | RBC mil/mm$^3$ | (HGB) g/dl | (HCT) % | MCV $\mu^3$ | MCH pg | MCHC g/dl | WBC mii/mm$^3$ | PLT mii/mm$^3$ |
|---|---|---|---|---|---|---|---|---|
| **Aver.** | 7.04 | 10.8 | **31.8** | **44.3** | 14.9 | 33.6 | 8.5 | **223** |
| **N.V.** | 6.5±1.4 | 10.2±1 | 35±3 | 53.4±2.2 | 16±2.5 | 29.1±2.1 | 8.0±1.5 | 1-8 |

*Table 3*

**Mean quantitative haematological examination in cattle in the GP - Group III Oil extraction zone areas from Băbeni and Mădulari (Cows with reproductive disorders)**

| Crt. Nr. | RBC mil/mm$^3$ | (HBG) g/dl | (HCT) % | MCV $\mu^3$ | MCH pg | MCHC g/dl | WBC mii/ mm$^3$ | PLT mii/mm$^3$ |
|---|---|---|---|---|---|---|---|---|
| **Aver.** | 6.50 | 9.5 | **28.7** | **44.2** | 14.5 | 31.7 | 9.5 | **218** |
| **N.V.** | 6.5±1.4 | 10.2±1 | 35±3 | 53.4±2 | 16±2.5 | 29.1±2. | 8.0±1.5 | 1-8 |

*Table 4*

**Mean quantitative haematological examination in cattle in the GP - Group IV - Hill and mountain areas -**

| Crt. Nr. | RBC mil/mm$^3$ | (Hb) Hemoglobin g/dl | (Ht) Hematocrit % | MCV $\mu^3$ | MCH pg | MCHC g/dl | WBC mii/ mm$^3$ | PLT mii/mm$^3$ |
|---|---|---|---|---|---|---|---|---|
| **Aver.** | 6.73 | 9.7 | **28.8** | **42.6** | 14.2 | 33.5 | 9.9 | **195** |
| **N.V.** | 6.5±1.4 | 10.2±1 | 35±3 | 53.4±2. | 16±2.5 | 29.1±2.1 | 8.0±1.5 | 1-8 |

Through analysis and synthesis of all data was actually aimed at establishing a diagnosis, identify risk factors for health and production, and developing an action plan for short and long term. Analysis (figure 1) of the values obtained by determining hematological parameters, diagnostics led to the conclusion that in these areas, there are effective at changing individuals, *microcytic normochromic anemia*, marked the first batch of animals and the other lot easier.

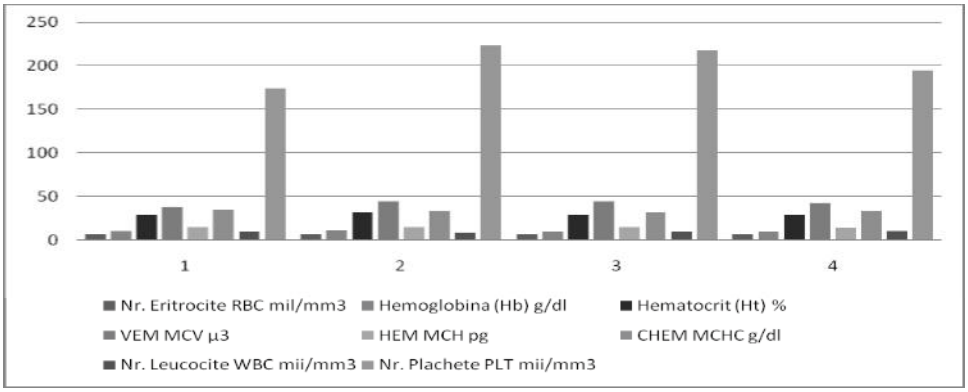


**Fig. 1. Haematological examination in cattle quantitative values of GP - comparative data between the 4 groups examined -**

The lactating cows are considered anemic when the number of erythrocytes falls below 5.1 million/mm$^3$, hemoglobin below 9.2 g/dl, haematocrit below 32%.

In cattle, unlike other species, being very diverse etiology of anemia, nutritional, toxic, parasitic, infectious, neoplastic, etc. This type of anemia can be found in chronic inflammation and infection piogene, hematopoietic marrow damage (eg. poisoning lead).

In the modern, myelopathy anemias are considered so that the bone marrow is more or less affected.

In lactating cows, a factor emphasized is that the values of red series (eritron) and especially of the hemoglobin and hematocrit are closely correlated with milk production. Also, the incidence of anemia is influenced by the lactation (Dumitru, 1996). For example, the daily production of up to 7 liters of milk anemia is found in 4.4% of animals at a yield of 8 to 16 liters, the incidence of anemia increased to 15.5 % (Parvu, 1992).

Another aspect to be highlighted is the number of platelets that is tens of times higher than reference values considered. The outcome was platelet count in conjunction with the microscopic observation of stained smears, which allowed the estimation of their approximate number, and on the other hand showed their morphology. Thus, the large number of platelets in the smear was confirmed by observation of the distribution and occurrence of so called „*beach platelet*" (platelet grouping in a very large number). These issues can lead to a state of hyper-coagulabilitate that can precede a thromboembolic syndrome.

Etiopathogenesis trombopatia (thrombocytosis, in our case) is complex and varied (eg, toxic, infectious, parasitic, anaphylactic dismetabolic, etc.) Hemostatic functions of the disorder can cause capillaries of platelets or plasma coagulation factors, with the participation their different degrees, rarely singular and often simultaneously (Williams, 2001). Between hemostasis management system and maintenance system in the liquid in blood vessels is a vital biological balance (homeostasis, hemostasis, and correlations between coagulation and anticoagulation system are more apparent than in physiology pathology).

Qualitative examination of the blood, performed on peripheral blood smear confirms dwindling number of red blood cells with hemoglobin and cargo, the quantitative aspects are accompanied by changes in the size, shape and color of erythrocytes, were seen as being in a higher percentage 20% on smear, *anisocytosis* issues (mycro and macrocytes) *poikilocytosis* (*acanthocytes*, *spherocytes*, *anulocytes*) and *anisocromia*, the smear is frequently present *anulocytes*, *polycromasia*. Eosinophilic reaction, on the other hand, parasitic existence beyond suspicion (confirmed by examination of the slaughterhouse and otherwise) in

cattle can criminalize the existence of allergies and even ovarian hypofunction, eg persistent corpus luteum (Radostits, 1994).

Quantitative or qualitative changes in these blood constituents is an indicator of health because they are the result of nutritional deficiencies, certain functional disorders or organic lesions. Changes detected are also the body's defense reactions, which can be interpreted as a consequence of the aggression of various factors including those emitters, nutritional and parasitic.

Lack of evidence of an epizootic investigation, such as data on animal nutrition, with involvement in the state parameters, was offset by the relatively high number of tests performed on animals established in the four groups, which were derived from blood samples. There were no data to indicate that cattle given diets were found responsible for causing imbalances in the interpretation of results in the field, especially that the cows were grazing period.

## 3. CONCLUSIONS

3.1. One major concerns time and directly affects the quality of life, is related to climate change, environmental pollution has become one of the most serious problems discussed and an outstanding contemporary society, with both short-term effects as well as long term;

3.2. It was found that in terms of pollution by pollutants in the atmosphere are most contaminated soils near sources of pollution, soil contamination depending on the level and rainfall regime, they usually wash the atmosphere pollutants and deposited them on the ground but also wash and soil, helping to conveying pollutants to the envoys;

3.3. Decreased hematocrit and mean corpuscular volume as expressed by changes anisocytosis cytomorphological examination, translate poikilocytosis, anisocromia and development of a *normochromic microcytic anemia* at the level of actual and individuals;

3.4. Anemia, with lymphocytic and eosinophilic reaction to translate the existence of a general adaptation syndrome due to action medulotoxic agents with vital economic and unfavorable prognosis; 3.5. It is essential to establish a program of regular evaluation of the territorial integrity of nutritional and metabolic indices of livestock through animal welfare, precise and measurable (hematological and biochemical tests);

3.6. Hematology can be a *byomarker*, metabolic disorders can discover long before their clinical expression, and prevent the growth and reproductive disorders, increasing the proportion of morbidity and

mortality, low production efficiency, etc.; 3.7. It is necessary to develop and implement an integrated program of monitoring the welfare of lactating cows in this area, both to increase the quality and quantity of milk production and dissemination of the results by livestock farmers in the prophylaxis.

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# EFFECT OF PROBIOTICS ON PERFORMANCE AND METABOLIC PARAMETERS IN SWINE

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**Keywords:** probiotics, pigs, hematologic and biochemical parameters, daily gain weight, mortality.

**SUMMARY**

The aim of the present study was to investigate the influence of probiotics, based on genus Bacillus and Lactobacillus in sows, suckling pigs and fattening pigs following the protocol: "L" Probiotical solution containing *Lactobacillus* was administrated to the new born piglets and "B" Probiotics premix containing *Bacillus*, which was administrated to pregnant and lactating sows and to fattening pigs in experimental groups. Control groups received no Probiotics.
The results were the significantly reduction of mortality caused by diarrhea in the piglets' experimental group in comparison with the control batch, the raise of the daily weight gain and the shortening of the fattening period.
The number of erythrocytes (RBC), leucocytes (WBC), hematocrit values (Hct) and hemoglobin concentration (Hb) were similar in both groups the differences being insignificant in sows, suckling piglets and fattening pigs.
The levels of total proteins (TP), albumins (Al) and gamma globulins (γ Glob) were significantly increased in the experimental groups in pregnant and lactating sows, suckling piglets and fattening pigs in comparision with control groups.

The use of antibiotic growth stimulators has been gradually eliminated in European Union Countries and things seem to continue that way for the treatment of bacterial intestinal or respiratory infections as well. The use of probiotics in nutritional therapy and prophylaxis has been aimed to counteract the main results of stress, disease or use of antibiotics (Pătrăşcanu, 2009; Rădoi, 2001). In literature, there is this new and spare trend of studying the beneficial metabolic effects of probiotics on fattening pigs and sows (Jeresiunas *et al*., 2006; Link *et al*., 2008, 2008) Various types of stress (as in nutritional, transport, birth, weaning, separating, bacterial, viral or micotic exposure) cause the inhibition of good digestive microflora, modifing the gastrointestinal pH, leading to imunosupression, pathogen microflora multiplication and, finally, the decline of the productive performances and even mortality (Rădoi, 2008).

## 1. MATERIAL AND METHOD

The research was conducted in a closed circuit pig farm in the South. Probiotic products obtained from various combinations of bacterial genus (*Bacillus, Lactobacillus, Enterococcus*) were administered to pregnant and lactating sows, piglets and fat pigs, by the following protocol:

- **„L" probiotic**, oral solution, containing Enterococcus faecium, Lactobacillus acidophillus, Lactobacillus casei and Lactobacillus plantarum, was administrated to new born piglets.
- Experimental batch: 600 piglets, 2 doses: 2 ml in the first day and 4 ml in the fourth day of life;
- Control batch: 600 piglets that received in the first and the fourth day of life the same amount of sterile physiological serum.
- **„B" probiotic**, containing *Bacillus licheniformis* and *Bacillus subtilis*, was administrated to pregnant and lactating sows and fat pigs ratio.
- Experimental batch: 50 pregnant sows in their one hundredth day of gestation, received 0,3 kg of probiotic premix/ ton until calving and 0,6 kg/ ton in the first two weeks of lactation;
- Control batch: 50 pregnant sows, without probiotics in food.
- Experimental batch: 400 fat pigs, 72 days old, fed on the first phase, to 95 days, with 450 g premix/ton and from 95 to 140 days with 150g premix/ton;
- Control batch: 400 fat pigs, normally fed, without probiotics, of the same age and environmental conditions as the experimental batch.

Concentration of „L" probiotic product: - 3,2 X $10^9$ UFC/ml solution.

Concentration of „B" probiotic product in sow's food:

- 3,2 X $10^9$ UFC/g, in food for lactant sows and 1,6 X $10^9$ UFC/g, in food for pregnant sows;
- 2,1 X $10^9$ UFC/g, in food for fat pigs in the first phase; and 0,8 X $10^9$ UFC/g, in food for fat pigs in the second phase.

Investigations were performed on average daily gain, mortality rate and causes of the newborn piglets losses at the end of the lot and after treatment with probiotic solutions. There have been intended weighing for fattening pigs, calculating average daily gain during 72-140 days of life.

Blood samples have been collected from pregnant and lactating sows, piglets and fat pigs and there were performed hematological tests: erythrocyte (RBC), leukocytes (WBC) hemoglobin (Hb), hematocrit

(Hct) and biochemical assay of protein status: total protein (Tot Prot), albumins (Alb), total globulins (Tot Glob) and gamma-globulin (γ Glob). For blood cell count (flow cytometry) and to determine biochemical parameters it was used the methodology from laboratory of clinical diagnosis of FMV Bucharest.

## 2. RESULTS AND DISCUSSIONS

The analysis of major changes in hematological parameters (RBC, WBC, Hb, Hct) showed similar values for experimental and control groups, the differences being insignificant.

Total protein (Tot Prot), albumins (Alb), total globulins (Tot Glob) and gamma-globulins (γ Glob) increased significantly in the experimental groups of sows compared with control groups, after the first 14 days of lactation (Tab. 1, 2).

***Table 1***

**Mean values of hematological and biochemical parameters in pregnant sows at day 100 at the beginning of the experiment**

| **Specification** | **Reference values** (Pârvu, 2003) | **Control batch** | **Experimental batch treated with "B" Probiotic** |
|---|---|---|---|
| **RBC ($10^6$/mm$^3$)** | 6.5±0.5 | 5.25±0.76 | 5.61±0.98 |
| **Hb (g/dl)** | 13.0±0.5 | 11.4±0.28 | 12.1±0.78 |
| **Hct (%)** | 40±5 | 37.8±3.01 | 39.1±2.31 |
| **WBC ($10^3$/mm$^3$)** | 15.2±2 | 14.8±2.01 | 15.66±2.4 |
| **Tot Prot (g/dl)** | 7-8 | 7.7±0.83 | 7.3±0.90 |
| **Alb (g/dl)** | 3.5±0.3 | 2.5±0.28 | 2.6±0.20 |
| **Tot Glob (g/dl)** | 4-4.5 | 5.2±0.44 | 4.7±0.31 |
| **γ Glob (g/dl)** | 1.7±0.2 | 1.89±0.50 | 1.89±0.39 |

***Table 2***

**Mean values of hematological and biochemical parameters in lactating sows in the 14-day**

| **Specification** | **Reference values** (Pârvu, 2003) | **Control batch** | **Experimental batch treated with "B" Probiotic** |
|---|---|---|---|
| **RBC ($10^6$/mm$^3$)** | 5.5±0.9 | 5.18±1.01 | 5.68±1.34 |
| **Hb (g/dl)** | 12.0±0.6 | 10.20±0.91 | 10.80±0.50 |
| **Hct (%)** | 39±4 | 36.60±3.48 | 38.40±2.56 |
| **WBC ($10^3$/mm$^3$)** | 16.7±2.04 | 11.35±2.5 | 11.80±3.4 |
| **Tot Prot (g/dl)** | 7.81±0.34 | 6.53±0.53 | 8.05±0.84 |
| **Alb (g/dl)** | 3.25±0.22 | 2.10±0.45 | 2.94±0.56 |
| **Tot Glob (g/dl)** | 4.56±0.28 | 4.43±0.35 | 5.11±0.63 |
| **γ Glob (g/dl)** | 1.50±0.90 | 1.65±0.60 | 2.40±0.50 |

For the sows in the experimental batch (in the last 15 days before birth and the first 4 weeks of the lactant period), the administration of „B" Probiotic pointed out a very important drop in mortality by comparison with the control batch, the difference exceeding the normal situation by 62,5% (see Table 3).

This is a serious argument for using probiotics and there is also the priority economic reason in order to increase the profit in swine farms.

***Table 3***

**Influence of "B" probiotic administration to pregnant sows on piglet mortality**

| Specification | Control batch | Experimental batch treated with "B" Probiotic | Diference | Procentual diference % |
|---|---|---|---|---|
| **Mortality in piglets** | 8 | 3 | 5 | 62.5 |

Effects of "L" probiotic administration on piglets in the first and 4th day of life consisted in a significant reduction in mortality caused by diarrhea and increasing of daily weight gain by 36 g over the whole period of infancy.

***Table 4***

**Influence of "L" probiotic administration on infant mortality due to diarrhea and daily weight gain in piglets**

| Specification | Control batch | Experimental batch treated with "L" Probiotic | Diferece |
|---|---|---|---|
| **Piglets mortality due to diarrhea (%)** | 3 | 0,5 | 2,5 |
| **Daily weight gain (Kg)** | 0.188 | 0.224 | 0.036 |

The changes in haematological parameters investigated showed no significant difference between the experimental group who received "L" probiotics and the **control batch**, in the category of piglets (14 days age).

The main biochemical parameters showed a significant increase for total protein (Tot Prot), albumins (Alb) and gamma-globulins (γ Glob) (Fig.5).

*Table 5*

**Mean values of hematological and biochemical parameters in infant piglets**

| Specification | Reference values (Pârvu, 2003) | Control batch | Experimental batch treated with "B" Probiotic |
|---|---|---|---|
| **RBC ($10^6$/mm$^3$)** | 5.4±1.2 | 4.9±1.6 | 5.3±1.9 |
| **Hb (g/dl)** | 11.6±0.8 | 10. 8±0.9 | 11.2±0.8 |
| **Hct (%)** | 38±3 | 37±4 | 39±5 |
| **WBC ($10^3$/mm$^3$)** | 18±5 | 16.2±2.4 | 19.5±3.9 |
| **Tot Prot (g/dl)** | 5.8±0.4 | 5.4±0.2 | 6.5±0.4 |
| **Alb (g/dl)** | 2.8±0.7 | 2.4±0.5 | 3.2±0.5 |
| **Tot Glob (g/dl)** | 3±0.5 | 3.0±0.4 | 3.3±0.6 |
| **γ Glob (g/dl)** | 1.70±0.2 | 1.8±0.4 | 2.4±0.3 |

In fat pigs, „B" probiotic was administrated in the first phase (from 72 to 95 days) to fat pigs of 28 to 40 kg and in the second phase (from 95 to 140 days), until the weight of 70 kg.

"B" Probiotic administration on fat pigs caused an increase in average daily gain, compared to the control group, with 41 g in the period 72-95 days, with 66 g in the period 95-140 days, 50 g respectively for the whole fattening period.

*Tabelul 6*

**Influence of "B" probiotic administration on daily weight gain in fat pigs**

| Parametri productivi | Control batch | Experimental batch treated with "B" Probiotic |
|---|---|---|
| **Daily weight gain I (g) 72-95 days** | 480 | 521 |
| **Daily weight gain II (g) 95-140 days** | 600 | 666 |
| **Total weight gain I+II (g) 72-140 days** | 560 | 610 |

"B" Probiotic administration in the experimental group of fattening pigs in the fodder resulted in an insignificant increase in hemoglobin (Hb), hematocrit (Hct) and leucocytes (WBC) compared with untreated control group. Total protein (Tot Prot) and albumins (Alb) values have risen very significantly (Tab. 7).

*Table 7*

**Mean values of hematological and biochemical parameters in fattening pigs**

| Specification | Reference values (Pârvu, 2003) | Control batch | Experimental batch treated with "B" Probiotic |
|---|---|---|---|
| **RBC ($10^6$/mm$^3$)** | 5.4±1.2 | 4.9±1.6 | 5.9±1.1 |
| **Hb (g/dl)** | 11.6±0.8 | 10.8±0.9 | 10.0±0.7 |
| **Hct (%)** | 38±3 | 37±4 | 38.4±3 |
| **WBC ($10^3$/mm$^3$)** | 18±5 | 16.2±2.4 | 21.6±5 |
| **Tot Prot (g/dl)** | 5.4±0.4 | 5.4±0.2 | 5.9±0.6 |
| **Alb (g/dl)** | 3.2±0.7 | 2.4±0.5 | 3.4±0.9 |
| **Tot Glob (g/dl)** | 2.2±0.6 | 3.0±0.4 | 2.5±0.9 |
| **γ Glob (g/dl)** | 1.5±0.9 | 1.48±0.7 | 1.91±0.9 |

## 3. CONCLUSSIONS

1.1. „B" Probiotic administration in pregnant sows' food (the last two weeks) and lactant (the first four weeks) led to 62,5% drop of mortality in piglets, in comparison with the control batch.

1.2. „B" Probiotic administration in fat pigs' food, in the first and the second phase, led to the rise of daily weight gain with 50g/day and reduces the fattening period.

1.3. „L" probiotic, oral solution, given to piglets in two doses, in the first and fourth day of life, significantly reduced the mortality caused by diarrhea and rised the daily weight gain by 36g/day.

1.4. The values of erythrocytes, leukocytes, hemoglobin, and hematocrit in all experimental groups (pregnant and lactating sows, piglets and fattening pigs) were similar to those of control groups of the same category.

1.5. Total protein, albumin, total globulin and gamma globulin levels were significantly increased in all experimental groups (pregnant and lactating sows, piglets and fattening pigs) compared with control groups.

1.6. The nutritional therapy and prophylaxis with probiotics, from Bacillus and Lactobacillus strains, affected the health and performances of sows, piglets and fat pigs, being an alternative method to antibiotics as growth factors.

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# STUDIES REGARDING QUALITATIVE DETECTION OF ANTIBIOTICS RESIDUES IN RAW MILK

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**Key words**: antibiotics, residues, raw milk.

**SUMMARY**

The purpose of this research was to evaluate the contamination degree of milk with antibiotics residues, antibiotics-free milk being a desiderate in dairy industry. Study material was represented by raw milk sampled from collecting centers and farms.
To accomplish this purpose, it was used a qualitative microbiologic test, which is easy to apply and read, in order to detect the eventual inhibitors in milk.
The obtained results were considered normal and promising considering the fact that lately it is obvious the interest of farmers and milk industry to obtain dairy products with less chemical residues, even ecological.

Milk and milk products, unlike other products of animal or vegetal origin, are subdued to a high number of analysis in order to know the chemical composition, to appreciate the alimentary value, to trace the forgeries and contaminations, to check the deviations from standard features, etc.

Monitoring some xenobiotics becomes more important as many studies showed the possibility that these chemical substances can penetrate internal barriers of the organism and eliminate in milk in risky values. Moreover, their presence was noticed also in milk products, due to the contamination of raw milk and also due to the contamination in the processing stages.

The adhesion of Romania to European Union required practically the reconsideration of the quality concept for consumption milk and raw milk.

In order to perform this study, there were collected milk samples from several collection centers and bovine farms near Bucharest which supply raw milk to some processing units

The methods used for the investigations were applied according to actual European standards.

In order to detect antibiotics residues in raw milk, it was used the protocol recommended by E.U. legislation, which requires that the detection of antibiotics in raw milk have to be done in two stages:

- the first stage is a qualitative microbiological procedure which allows the identification of many antibiotics used in veterinary

practice, with a high risk to contaminate the milk: penicillin, ampicillin, cloxacillin, nafcillin, tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, dihydrostreptomycin, neomycin, kanamycin, bacitracin, erythromycin, rifampicin and some sulfamides;

- the second stage is the confirmation of the results obtained after the qualitative microbiological test.

## 1. MATERIALS AND METHODS

Qualitative detection of antibiotics residues in milk samples was performed using a method established as standard in E.U. (Commission Decision 91-180, 1991). Milk samples were analyzed immediately after the collection, so the preliminary procedures consisted in successive homogenizations by the overthrowing the recipients for 2-3 times and then the samples were left at rest for 1-3 minutes in order to eliminate air bullas.

The microbiological procedure was based on the use of a *Bacillus stearothermophilus*, var. *calidolactis* bacterial strain (ATCC 10149) as test microorganism. This test allows the detection of raw milk samples which contain antibiotics over the admitted limits.

The principle of the method consisted in the adding of milk samples over gelose, which contains a pH indicator and *Bacillus stearothermophilus* var. *calidolactis* spores (ATCC 10149); this strain has a very good sensitivity, especially for penicillin. Normal growth of this bacteria and acid synthesis after incubation produce the change of pH indicator from purple to yellow. If milk sample contain inhibitory substances, then the color of pH indicator remains unchanged (purple).

The objective of this research was to evaluate the contamination degree of milk with antibiotics residues.

There were used commercial tubes that contain 50 microliters of nutritive mix: dregs extract, glucose, soluble starch, bromcresol purple and water, along with *Bacillus stearothermophilus* spores. After mixing the milk samples, 0.1 ml milk was transferred in the test tubes. There were also prepared positive controls (using a penicillin standard solution 4 ppb) and negative controls (using milk known to be free of antibiotics). The tubes were closed with corks and they were introduced in a water bath at 64°C for 2½ - 2¾ hours. After that interval, it was studied the color of the gelose medium.

A purple color of the medium in the tubes containing positive control (penicillin) or milk samples indicates the presence of antibiotics

of sulfamides; the sensitivity of the method is considered appropriate if the tubes with standard penicillin solution remain purple.

A partially purple coloration in the tubes with milk samples indicates an uncertain or doubtful result.

A yellow coloration of the tubes containing control milk (without inhibitory substances) or sample milk indicates the absence of the inhibitory substances for the tested microorganism.

If all tested tubes, including negative control, are purple, then it is considered that the tubes do not contain viable spores and the analysis have to be redone.

This method qualitative detects antibiotics in milk samples, its sensitivity comparatively to maximum admitted limits being presented in table 1.

***Table 1***

**The detection limits of qualitative microbiological test and maximum admitted limits for antibiotics in milk (Commission Decision 2377/90)**

| **Antibiotic** | **Detection limit (ppb)** | | **Maximum admitted limit (ppb)** |
|---|---|---|---|
| | *Negative* | *Positive* | |
| Penicillin | 2 | 6 | 4 |
| Ampicillin | 2 | 5 | 4 |
| Cloxacillin | 15 | 35 | 30 |
| Nafcillin | 6 | 11 | 30 |
| Tetracycline | 100 | 400 | 100 |
| Oxytetracycline | 200 | 450 | 100 |
| Chlortetracycline | 150 | 500 | 100 |
| Chloramphenicol | 7000 | 15000 | Zero tolerance |
| Dihydrostreptomycin | 4000 | 13000 | 200 |
| Neomycin | 1000 | 22000 | 1500 |
| Kanamycin | 9000 | 28000 | 150 |
| Bacitracin | 60 | 140 | 100 |
| Erythromycin | 1000 | 2250 | 40 |
| Rifampicin | 10 | 140 | 60 |

## 2. RESULTS AND DISCUSSIONS

Study material was represented by milk samples collected from five collection centers and two farms near Bucharest. The researches were performed between 2006-2009 on 1423 milk samples.

The obtained results after the applying of identification tests for antibiotics residues are presented in table 2.

*Table 2*

**The results of qualitative microbiological test for the detection of antibiotics residues**

| Year | Analyzed samples | | Positive samples | | Doubtful samples | | Negative samples | |
|---|---|---|---|---|---|---|---|---|
| | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* |
| 2006 | 421 | 100 | 20 | 4.75 | 21 | 4.99 | 380 | 90.26 |
| 2007 | 378 | 100 | 16 | 4.23 | 17 | 4.50 | 345 | 91.27 |
| 2008 | 397 | 100 | 14 | 3.53 | 15 | 3.78 | 368 | 92.69 |
| 2009 | 227 | 100 | 10 | 4.40 | 11 | 4.85 | 206 | 90.75 |
| Total | 1423 | 100 | 60 | 4.22 | 64 | 4.50 | 1299 | 91.28 |

In 2006, there were analyzed 421 milk samples, from which 20 samples (4.75%) were positive, 21 samples (4.99%) were doubtful, while 380 samples (90.26%) were negative.

In 2007, there were analyzed 378 milk samples, from which 16 samples (4.23%) were positive, 17 samples (4.50%) were doubtful, while 345 samples (91.27%) were negative.

In 2008, there were analyzed 397 milk samples, from which 14 samples (3.53%) were positive, 15 samples (3.78%) were doubtful, while 368 samples (92.69%) were negative.

In 2009, there were analyzed 227 milk samples, from which 10 samples (4.40%) were positive, 11 samples (4.85%) were doubtful, while 206 samples (90.75%) were negative.

Globally, between 2006-2009 there were analyzed 1423 milk samples, from which 60 samples (4.22%) were positive, 64 samples (4.50%) were doubtful, while 1299 samples (91.28%) were negative.

The presence of antibiotics residues in milk suggests that these drugs are frequently used for prevention and treatment of mastitis and other infectious diseases in cows. Antibiotics, administered by any route, pass in milk and determine problems in the obtaining of fermented milk products and also upon consumers' health.

In this context, antibiotics-free milk represents a desiderate in dairy industry. Lately, there were developed many methods of milk analysis in processing units and in farms. Qualitative microbiological test is used to identify inhibitors in milk, being easy to apply and read; though, this test doesn't allow the identification of antibiotics (their group) and neither their quantity.

The presence of antibiotics in milk is wide-spread all over the world; several studies showed that on some milk markets, antibiotics residues are present in milk in 8-15%. Chung *et al.* (2009) identified 21 samples contaminated with antibiotics after the analysis of 260 milk samples,

which represents 8.1% positive samples. Khaskheli *et al.* (2008), after the analysis of 137 milk samples using qualitative microbial method in plates with *Bacillus subtilis*, identified 87 samples (63.5%) as negative, while 50 samples were positive (36.5%), with inhibition areas between 8.91±0.37 mm. Ammar *et al.* (2008) showed that average proportion of antibiotics residues in milk samples collected from different farmers was 22.2%.

Regarding these data, the results that we obtained can be considered normal and promising, in the context of the high interest for less contaminated and even ecological products.

## 3. CONCLUSIONS

3.1. Antibiotics-free milk is a desiderate in dairy industry.

3.2. Generally, from the 1423 studied milk samples between 2006-2009, 1299 samples (91.28%) did not contain antibiotics residues.

3.3. The obtained results after qualitative microbiological test place themselves between the general limits described in the specialty studies.

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# THE IDENTIFICATION OF SOME FALSE-POSITIVE REACTIONS FOR ANTIBIOTICS RESIDUES IN RAW MILK

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**Key words**: antibiotics, residues, raw milk, false-positive reactions.

**SUMMARY**

Some studies in the specialty literature pointed out the possibility that some samples detected as positive or doubtful after the applying of a qualitative microbiologic test in order to detect antibiotics residues in raw milk actually to manifest a false-positive reaction due to the interference of some domestic antimicrobial agents (such as lactoperoxidase, lactoferrin or lysozyme), somatic cells increased numbers or free fatty acids. Also, the possibility of milk contamination with chlorine after the sanitation of milking equipments can be bound to these false-positive results.
The studied material was represented by milk samples with positive and doubtful results after the applying of a qualitative microbiologic test, the confirmation being made after thermal treatment of these samples at 82°C for 10 minutes. The applying of this method led to the decrease of positive and doubtful results.

As future prospective, there is a continuous preoccupation for obtaining high-quality food products, with a low amount of chemical xenobiotics, fact that involves the setting-up of some specific measures.

The consequence of residues' presence in the animal organism and in the animal origin food products represents an actual subject in the conditions of the higher diversity of drugs used in veterinary therapy (Savu and Georgescu, 1999).

Some studies in specialty literature showed the possibility that some positive or doubtful results, consecutively using qualitative microbial test, were false positive reactions cause by the interference of some antimicrobial agents as lactoperoxidase, lactoferrin or lysozyme, by high number of somatic cells or by the presence of free fatty acids. At the same time, there is also the possibility of contamination of milk with chlorine after various products were used in cleaning milking equipment, a phenomenon that could lead to such results.

## 1. MATERIALS AND METHODS

The study material was represented by 60 positive and 64 doubtful results at microbial qualitative test (total: 124 samples); confirmation was performed by heat treatment at 82 ° C for 10 minutes.

## 2. RESULTS AND DISCUSSIONS

The obtained results are shown in table 1.

***Table 1***

**The results of heat treatment applied to positive and doubtful samples**

| Year | Samples | | Positive samples | | | | | | Doubtful samples | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Initially positive samples | | Confirmed samples | | lse positive samples | | nitially doubtful samples | | Confirmed samples | | False positive samples | |
| | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* |
| 2006 | 421 | 100 | 20 | 4.75 | 18 | 4.28 | 2 | 0.48 | 21 | 4.99 | 4 | 0.95 | 17 | 4.04 |
| 2007 | 378 | 100 | 16 | 4.23 | 14 | 3.70 | 2 | 0.53 | 17 | 4.50 | 4 | 1.06 | 13 | 3.44 |
| 2008 | 397 | 100 | 14 | 3.53 | 12 | 3.02 | 2 | 0.50 | 15 | 3.78 | 4 | 1.01 | 11 | 2.77 |
| 2009 | 227 | 100 | 10 | 4.40 | 8 | 3.52 | 2 | 0.88 | 11 | 4.85 | 3 | 1.32 | 8 | 3.52 |
| Total | 423 | 100 | 60 | 4.22 | 52 | 3.65 | 8 | 0.56 | 64 | 4.50 | 15 | 1.05 | 49 | 3.44 |

In 2006, following the use of microbial qualitative test, 20 (4.75%) samples were considered positive and consecutively the heat treatment were confirmed 18 (4.28%) of them, another 2 samples (0.48%) were considered false negative. In 21 (4.99%) samples considered dubious, were confirmed 4 of them (0.95%) and 17 were false negative (4.04%).

In 2007, there were identified 16 (4.23%) positive samples and after the application of heat treatment there were confirmed 14 (3.70%), the remaining 2 (0.53%) samples were considered false positive reaction. In case of the 17 (4.50%) samples with doubtful results, there were confirmed 4 of them (1.06%), 13 (3.44%) samples were considered false positive reactions.

In 2008, out of 14 (3.53%) positive samples were confirmed 12 (3.02%) samples and 2 (0.50%) samples were considered false positive reaction. Another 15 (3.78 %) samples, there were confirmed as dubious reactions 4 (1.01%) samples, the remaining 11 (2.77%) samples were considered false positives.

In 2009, there were identified 10 (4.40%) positive samples and 8 (3.52%) samples confirmed, the other two (0.88%) samples were considered false positives. There were 11 (4.85%) suspicious samples and confirmed 3 of them (1.32%), the rest 8 (3.52%) samples were considered false positive reaction.

In the whole study period, out of 60 (4.22%) positive samples were confirmed 52 (3.65%) samples, 8 (0.56%) samples were false positive results. From 64 (4.50%) samples, which were initially dubious, a number of 15 (1.05%) samples were confirmed as positive, while 49 samples (3.44%) were false positive reactions.

The total of confirmed samples (positive and doubtful) and the false positive samples is shown in table 2.

***Table 2***

**Heat treatment applied to positive and doubtful samples – centralized results**

| Year | Samples | | Positive confirmed samples | | | | | | False positive samples | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | From initially positive samples | | From initially doubtful samples | | Total | | From initially positive samples | | From initially doubtful samples | | Total | |
| | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* | *Nr.* | *%* |
| 2006 | 421 | 100 | 18 | 4.28 | 4 | 0.95 | 22 | 5.23 | 2 | 0.48 | 17 | 4.04 | 19 | 4.51 |
| 2007 | 378 | 100 | 14 | 3.70 | 4 | 1.06 | 18 | 4.76 | 2 | 0.53 | 13 | 3.44 | 15 | 3.97 |
| 2008 | 397 | 100 | 12 | 3.02 | 4 | 1.01 | 16 | 4.03 | 2 | 0.50 | 11 | 2.77 | 13 | 3.27 |
| 2009 | 227 | 100 | 8 | 3.52 | 3 | 1.32 | 11 | 4.85 | 2 | 0.88 | 8 | 3.52 | 10 | 4.41 |
| Total | 1423 | 100 | 52 | 3.65 | 15 | 1.05 | 67 | 4.71 | 8 | 0.56 | 49 | 3.44 | 57 | 4.01 |

Analyzing these data, we concluded:

- in 2006 were confirmed a total number of 22 (5.23%) samples and 19 (4.51%) were false positive reactions;
- In 2007 were confirmed 18 (4.76%) samples and 15 (3.97%) presented false positive reactions;
- In 2008 were confirmed 16 (4.03%) samples and 13 (3.27%) were false positive;
- In 2009 were confirmed 11 (4.85%) samples but 10 (4.41%) were false positive reactions.

In the whole study period, there were 67 (4.71%) confirmed positive samples and 57 (4.01%) were considered false positive reactions.

In specialty literature, there are some others authors that showed this possibility of obtaining false positive results after applying the microbial qualitative test. Tyler *et al.* (1992) identified 45% false positive samples from milk samples collected from cows with mastitis experimentally induced by endotoxin. Van Eenennaam *et al.* (1993) observed that in case of naturally occurring mastitis were identified 37.7% false positive reactions. Mixing milk in the tank obviously lead to the dilution of the milk from cows with different forms of mastitis in which the number of natural inhibitors is usually higher.

There have also been studies that showed that the percentage of false-positive results is higher in milk from small ruminants, due to their high content of natural inhibitors, which is not caused by the infection factors. The same authors mention that in case of small ruminants, increased frequency of false-positive samples is related with the end of lactation, and also, another cause might be preserving the milk with

sodium azide. In the present study, the samples were analyzed immediately after sampling, without the need of chemical preservation.

The method of heat treatment at 82°C for 10 minutes of positive and doubtful samples can be useful to eliminate false-positive results from the calculation of the final statistics. Kosikowski and O'Leary (1963) stated that by using this heat treatment there were successfully neutralized all natural inhibitors of 11 milk samples, the subsequent application of plate microbiological methods leading to negative results.

## 3. CONCLUSIONS

3.1. Application of heat treatment for the positive and doubtful samples led to a decreased percentage of samples in both cases.
3.2. Samples heat treatment can be an easier way to eliminate false positive reactions.
3.3. Heat treatment method can be applied without restrictions, positive reactions not being affected.

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# UNIVERSITY EDUCATION AT EUROPEAN LEVEL IN THE FIELD OF VETERINARY MEDICINE

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**Key words:** veterinary education**,** new curricula, labour market demands

**SUMMARY**

The objectives of veterinary medical education institutions are the provision of adequate veterinary training, ethics and science-based, which allows graduates to practice veterinary profession in all recognized areas of veterinary medicine. The project aims to increase the relevance and compatibility of undergraduate curricula in relation to labor market demands and to the changes induced by the knowledge society through developing new curricula and improving the internal quality assurance in higher education institutions of veterinary medicine at the sectoral level.

## PROJECT PARTNERSHIP

University of Agronomical Sciences and Veterinary Medicine Bucharest

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad" Iasi

Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara

Shotron Association

## PROJECT PRESENTATION

Initial quality of human resources is the precondition for performing a competitive labor market. A highly skilled workforce is essential to a knowledge-based competitive and sustainable economy. The objectives of veterinary medical education institutions are the provision of adequate veterinary training, ethics and science-based, which allows graduates to practice veterinary profession in all recognized areas of veterinary medicine.

The project aims to increase the relevance and compatibility of undergraduate curricula in relation to labor market demands and to the changes induced by the knowledge society through developing new

curricula and improving the internal quality assurance in higher education institutions of veterinary medicine at the sectoral level.

## PROJECT OBJECTIVES

The specific objectives are:

- Develop / implement a modern curriculum, which meets the CNCSIS methodology/ European requirements in veterinary care and a system of e-learning - to modernize the teaching learning process for 2500 students enrolled in veterinary medical education system;
- flexible learning opportunities to the students in the undergraduate program of study by developing an online learning community to provide resources and materials to those interested in innovative digital veterinary medicine;
- develop and implement an integrated quality management system to support quality assurance at institutional level, as required by EU veterinary medical education system;
- strengthen professional skills of 80 people, teaching staff involved in developing modern curricula and adapted to European veterinary medical education system;
- strengthen professional skills of 20 people, teaching staff involved in quality assurance at higher education institution in the field of veterinary medicine through peer learning activities with colleagues from other universities in the field.

## PROJECT RESULTS

- 1 operational vocational training program for staff involved in curriculum development to improve the academic veterinary medical skills in designing and developing the curricula
- 80 people - staff involved in the development of university programs participating in the program of veterinary medical professional
- A modular curriculum with four new modules complement the ongoing program of undergraduate studies for veterinary specialization developed / piloted
- An e-learning system designed to support 2,500 students from the four universities

- 20 members of quality assurance committees at university, participants in four meetings with the takeover of peer learning best practices organized / conducted
- An integrated system of quality management tools, support materials for information, forums, evaluation reports, statistics, questionnaires developed / tested / validated technical
- Providing e-learning room with 1 laptop, 26 computer, 1 server and furniture for 26 persons
- 3 workshops between the universities partners in the project
- 3 seminars with the representatives of the sector institutions, for promoting and for dissemination of the project results

## ADDED-VALUE ELEMENTS

- Extension of e-learning curriculum offers to allow a better preparation of students through access to virtual information resources and interactive communication, and flexible learning opportunities and to attract a more diverse student population
- Promote examples of best practices both among students and between staff involved in the development of university curricula and digital resources by establishing a virtual community that allows saving time and resources allocated for initial training
- Strengthen professional skills of personnel involved in the development of university curricula, the development of modern curricula and tailored European veterinary medical education system using digital resources
- Strengthen university autonomy, coupled with the introduction of a national quality assurance system, assessing both external and internal, as well as measures of institutional quality management - prerequisite for professional employment and improve European competitiveness in the field of veterinary medicine.

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Priority Axis 1 – "Education and professional training supporting economical growth and knowledge based society development".

Key Area of Intervention 1.2. "Quality in university education".

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# PUBLICATION IN THE ELECTRONIC MEDIUM OF SCIENTIFIC ACADEMIC RESEARCH RESULTS IN THE CONTEXT OF INTELLECTUAL PROPERTY, COPYRIGHT AND RELATED RIGHTS

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**Key words**: academic research, scientific output, copyright, knowledge production, intellectual property context, open access, informational electronic medium

**SUMMARY**

The main purpose of research is to produce new knowledge. Scientific papers, such as articles in specialized journals, proceedings, articles in reference works, scientific blogs, Institutional Repositories, act as lucrative form of this new knowledge. Academic publishing (scientific) describes a system that uses necessarily a formalized subsystem of scientific review and makes information available for large academic audiences. This system varies relating to results, organization and competences from one scientific domain to another. The paper considers legal constraints on the publication as open access of works covered by copyright which must fully comply with intellectual property rights, respectively copyright law and to reward the authors correctly. In this context it is emphasized the importance of legislation designed to ensure an adequate legal framework for intellectual property rights of authors and involvement of European and national legislative bodies in this direction. It is emphasized the importance of legal provisions on copyright, developed in a flexible manner to foster development, creation and exploitation of works creative content.

## THE EUROPEAN VISION ON DIGITAL CONTENT, OPEN ACCESS TO SCIENTIFIC PRODUCTION AND COPYRIGHT

1. Preconditions for the creation, access and use of infodocumentary electronic materials

The main purpose of scientific research regardless of the runs is to produce new knowledge that can lead, on different levels of use of course, to a practical end. Scientific papers, such as articles in branched journals, scientific communication ("Proceedings"), articles in reference works, scientific blogs, electronic institution databases ("institutional repositories") play the role of lucrative form of this new knowledge.

Today, the usage of digital information technologies foster the flow of communication for results in the scientific research within regional and international community of academic research. The actual dimension of the development of electronic digital environment in academic infodocumentary structures and research environments shows the effectiveness and usefulness of this modern environment.

An important aspect of this environment is the research based on open access to information, that actually requires equal access for any individual to scientific information. There are two models for open access to information. One is when a publisher decides to offer free access to information published by him, and the second model also called open archives refers to a situation where an author or an organization producing information choose to distribute it chargeless (by removing the role of publisher) to all those interested. The second model is exposed to a high risk of proliferation of distorted information, without being peer-reviewed and may contain critical errors.

Anything that addresses electronic sources of scientific information should consider their link with academic community in general and specifically with academic research. It remains to be demonstrated whether this link is a direct one and enriched access to electronic sources of scientific information leads to increased performance in academic research.

In terms of copyright, these are all prerogatives empowering the authors on the works they have created and the institution of copyright is the instrument by which the creators and their works are protected[1]. Literary and artistic works are protected by the *Berne Convention for the Protection of Literary and Artistic Works*, which dates from 1886, as revised in 1971. Berne Convention provides that a work is protected by copyright as long as "*intellectual property of a literary, artistic or scientific work corresponds to an author for the simple fact that he created it*" and "*there are considered objects of intellectual property all literary, artistic and scientific original creations expressed through any medium and in any format, tangible or intangible, known or to be invented in the future*."[2]

In 1996 the World Intellectual Property Organization (WIPO) drawn up two so-called *Internet treaties*. We refer to the *WIPO Copyright Treaty (WCT) and WIPO Performance and Phonograms Treaty (WPPT)*, which, in the period 1996-2002 have been ratified by 30 states, the minimum required by the United Nations to become effectual. In this way it is updated the Berne Convention and there are introduced the new digital environment elements, which legally established the legal position on intellectual property rights of authors in this virtual area.

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[1] *DREPTURI de autor. http://ro.wikipedia.org/wiki/Drepturi_de_autor* . Accessed 15.09.2010.

[2] *CONVENȚIA de la Berna pentru protecția Operelor Literare și Artistice http://ro.wikipedia.org/wiki/Drepturi_de_autor.* Accessed 15.09.2010.

Today, in the process of developing the Information Society in Europe, for the development and use of digital information content we are using the same intellectual property laws and copyrights as for all other original creations. The Copyright protection opened the door to intense debate with the massive growth of the Internet in the early '90s and spread of digital technologies. The objective cause of these discussions has been the easy access to and the reproduction of digital content, in whatever form (text, image, video or sound). Compared with previous analogical period which raised difficulties in reproduction due to "binding" of the content with the format on which was inscribed, the current digital environment no longer has the same restriction.

It therefore puts emphasis on the development of digital information environment for digital preservation and to provide information in a manner most appealing to the public. One of the flagship projects of the initiative *i2010 – EU Policy Framework for Information Society and Media*[3], being considered an important initiative for information and communication technologies (ICTs) in the context of the Lisbon Strategy, is the *European Digital Library*. European Digital Library is a meta-portal linking to existing digital libraries in the countries of the European Union. These libraries provide material that was digitized or material produced directly in digital format.[4]

In this context of the European development of digital media, Viviane Reding, European Commissioner for Information Society and Media[5] said during talks with representatives of Google (September 2009) that the slow pace of the wide process of digitization began in Europe and the disadvantage created by their own rules of copyright could cause disturbance in the cultural future of Europe. Viviane Reding supported by other politicians in Brussels continues to advocate for the development of European digital heritage in order to optimize the pace of realization of this important project.

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[3] *i2010 – A European Information Society for growth and employment* http://ec.europa.eu/information_society/eeurope/i2010/index_en.htm Accessed: 15.09.2010.

[4] http://ec.europa.eu/information_society/activities/digital_libraries/index_htm Accessed 16.09.2010.

[5] *PROIECTUL bibliotecii digitale Google*. http://euractiv.ro/uniunea-europeana/articles|displayArticle/articleID_18168/Proiectul-bibliotecii-Google.html Accessed 16.09.2010.

## 2. ANALYSIS AND OPTIMIZATION OF THE SYSTEM OF COPYRIGHT IN THE EU

The problems generated by the interaction between European law on copyright and political framework for the development of digital media in Europe determine European officials involved in this area of digital technology development to apply a penetrating analysis of the existing copyright system in the European Union. In this connection the following questions are raised:

- The current legislative framework is sufficient for developing digital age?
- The current legislative framework provides access to digital content developed for users across Europe?
- The current legislative framework does ensure a fair reward for authors?
- The current legislative framework ensures a foundation for uniform digitization across Europe or there will persist fragmentation induced by national borders?
- Current EU legislative framework supports increasing digitization compared with efforts in this area on other continents?
- European copyright framework is sufficiently flexible and modern to offer the possibility of digitizing the "orphan" works[6] and those that are no longer printed?

There are obvious multiplication and diversification of creative, scientific production and current exploitation directions, derived naturally from the development of modern technologies. Regarding intellectual property protection, even if not need to develop new concepts, however, the current law on copyright and related rights needs to be amended and supplemented. This is necessary for a proper reflection of economic realities which refers to new forms of operation supported by modern information and communication technologies.

We analyze a demarche of European Parliament and EU Council, resulted in the development of Directive 2001/29/EC of the European Parliament and EU Council on the harmonization of certain aspects of copyright and related rights in the information society.[7] The purpose of

[6] Orphan works are those works which are still in copyright but whose owners can not be identified or located.

[7] *DIRECTIVA Parlamentului European și a Consiliului 2001/29/CE de armonizare a anumitor aspecte privind dreptul de autor și drepturile conexe în societatea informațională.*

this directive is to help the implementation of internal market freedoms, referring to observance of fundamental principles of property rights, including intellectual property, freedom of expression and public interest. By providing a harmonized legal framework on copyright and related rights that is intellectual property protection, the European regulatory approach aimed at encouraging investment in creativity and innovation, promote and develop competitiveness.

It was intended to preserve a fair balance between the rights and interests of rights holders and between them and the rights of users of works protected by copyright. The proposed harmonization at Community level recommended reconsideration in the light of the new electronic environment of legal actions brought by Member States at national level, to meet current technological challenges and enhancing cross-border exploitation of intellectual property.

This concern of European legislators highlights the importance of Union's legislation designed to ensure an adequate legal framework for intellectual property rights of authors. The legal provisions on copyright were basically designed to stimulate the development, creation and exploitation of creative content.

Another important milestone on copyright in Europe that is submitted to our attention is consultation document adopted by the European Commission (July 16, 2008), *Green Paper: Copyright in the Knowledge Economy*[8]. This consultative document is one of great importance in this area as an action taken in order to lead to a new directive on copyright and related rights, or to change and amend the existing Directive (*Directive 2001/29/EC of the European Parliament and EU Council on the harmonization of certain aspects of copyright and related rights in the information society).* The adopted Green Paper creates an opportunity for substantial changes to the exceptions relating to educational institutions, public libraries, archives, etc., through an advisory action by all stakeholders in the European Union, the ultimate goal being to clarify, modify, amend, repeal of parts of the current Directive in terms of information society and electronic environmental factors' evolution.

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http://www.legi-internet.ro/fileadmin/editor_folder/pdf/DIR29RO_IER.pdf. Accessed 16.09.2010.

[8] *CARTEA verde – Drepturile de autor în economia cunoasterii.* http://ec.europa.eu/internal_market/copyright/copyright-infso/copyright-infso_en.htm Accessed 20.09.2010.

In the following we present three important aspects of the Green Paper on how to best disseminate online content of works on *science, education and research*:

3. copyright exceptions for libraries and archives;
4. provision of digitized works;
5. digitization of orphan works problem.

**Exceptions to copyright for libraries and archives**

Current legislation relating to copyright provides two exceptions narrowly formulated in *Directive 2001/29/EC of the European Parliament and EU Council on the harmonization of certain aspects of copyright*[9] for libraries, educational institutions, archives and museums:

1. *An exception to the right of communication* to the public and *the right of making available* for research or private study by means of special terminals located on the establishments of such institutions (Art. 5, para. 2, lit. C of the *Directive 2001/29/EC*).

2. *An exception to the reproduction right* for specific acts of reproduction for noncommercial purposes (art. 5, para. 3, lit. N of *Directive 2001/29/EC*).

The *Green Paper: Copyright in the knowledge economy*[10] brings in the debate on two major issues related to libraries and other related institutions:

- making digital copies of materials held in library collections;
- using electronic means of distributing digital content to users.

In the infodocumentary structures area and beyond, is well known that the digitization of information materials, audiovisual and other content can serve a dual purpose: electronic distribution of digital content to Internet users and content preservation of digitized materials for future generations. The current legal framework does not provide libraries or archives for an exemption from the right of reproduction. Reproductions are only allowed in specific cases and for the preservation of works contained in library catalogs. The exception for libraries contained in the Directive and national rules implementing it are not always clear on the number of copies that can be made under this exemption or convert the format. Tuula Haavisto stresses in this connection that copying limits are set by copyright law and that they

[9] *Op.cit.* http://www.legi-internet.ro/fileadmin/editor_folder/pdf/DIR29RO_IER.pdf Accessed 20.09.2010

[10] *Op. cit.* http://ec.europa.eu/internal_market/copyright/copyright-infso/copyright-infso_en.htm
Accessed 20.09.2010.

vary from country to country. These limits refer to copying for educational, personal, research purposes, duplication of copies in libraries and archives for the persons with special needs, etc.[11]

Ambiguous situation in legal terms of digital media has led libraries and other public institutions to express increasingly deeper concerns in preserving digitized works, especially digital collections content transmission over the Internet.

Under current law on copyright *Provision of digitized works* there are under exception to the right of communication to the public and to the right of making available to the public works or other subject matter, provided that such communication or making available take place in the purpose of research or private study in the building area of institutions (art. 5, para. 3, lit. n of *Directive 2001/29/EC*). This exception *does not include* the electronic transmission of documents to remote end users and more than that, paragraph 4 of the Directive states that the exception for libraries and archives should not include *use in the context of online delivery of works or other protected objects*.

## CONCLUSIONS

Aspects of the *Green Paper: Copyright in the Knowledge Economy* which promote debate on how to best disseminate **online knowledge for research, science and education** have sought to launch an advisory cycle with all stakeholders for the union, aimed at identifying legal issues concerning copyright and related rights in the knowledge economy. In this regard, libraries, archives, educational institutions, and other concerned institutions were also and are invited to participate in the extensive consultation process with their own ideas and visions.

In conclusion of this review on some important steps of the legislative European bodies for intellectual property rights of authors, we summarize some key issues included in the Green Paper, for which was launched the invitation to submit views and comments at professional and institutional levels:[12]

- appropriateness of maintaining copyright exception for libraries and archives, since the publishers themselves provide online access to

[11] *DOCUMENTE audiovizuale şi multimedia în biblioteci: ghid IFLA.* Traducere şi adnotare de Mircea Regneală. Bucureşti: A.B.R., 2008, p. 141.

[12] *CARTEA verde: Drepturile de autor în economia cunoaşterii.* http://ec.europa.eu/internal_market/copyright/copyright-infso/copyright-infso_en.htm Accessed 22.09.2010.

their catalogs;

- to increase access to works within public libraries, educational institutions, museums and archives by entering into licensing agreements with publishers;
- examples of licensing schemes enabling online access to library collections;
- need to clarify the area for applying the exception for public libraries, schools, museums and archives in relation to:

a. format conversion;

b. the number of copies that can be made under the exception;

c. scanning of entire collections held by libraries;

3 need for legislation to clarify whether the scanning of works held by libraries in order to make them available on the Internet goes beyond the scope of current exceptions to copyright;

4 need for a new legal Community instrument on the issue of orphan works, which exceed the scope of Commission Recommendation 2006/585/EC of 24 August 2006;

5. this tool will be created by amending the 2001 Directive on Copyright in the Information Society or will act independently?

6. how to deal with border issues related to orphan works to ensure that EU-wide recognition of the solutions adopted in different Member States.

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# OPTIMIZATION OF METHOD SEPARATION LYMPHOCYTES FROM CATTLE AND SHEEP BLOOD

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**Key words:** optimization, assimilation, implementation, Percoll, Ficoll.

**SUMMARY**

The aim of this study was the optimization of methods for lymphocytes separation from the blood samples (cattle, sheep), taken on anticoagulant (Lithium-Heparin) in order to evaluate the immune response of animals by quantification of lymphocytes, their functional level, the functional differentiation of lymphocytes in effector cells. Then, we trying to reflect all this studies in assimilation and implementation of the test for lymphocytes stimulation in pure culture for paratuberculosis diagnosis.We used two methods which allowed the separation of different cells fractions from anticoagulant–treated blood, by centrifugation in density gradient, with appropriate culture media and compatible with living systems: Ficoll, Percoll.
This investigation reveal that we can not achieve the separation of lymphocytes in density gradient with Ficoll, Percoll medium, using anticoagulant –treated blood taken without diluation. The best results we obtain by blood diluation with an equal volume of RPMI 1640 medium and separation using the Percoll medium with 1,075 density (20°C-1800rpm, for 20 minutes; 20°C-2800rpm, for 20 minutes) of 3 ml of blood diluted ½ with RPMI 1640 and this will be added carefully in 3 ml Percoll 1,075.

Paratuberculosis (Johne`s disease) is a chronic enteritis of ruminants caused by Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis) and continue to present major problem for human and animals health.

The diagnosis of paratuberculosis is divided into two parts: the clinical diagnosis and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.

Immunological investigations must allow fast confirmation a clinical suspicion, but especially, to track infected animals not only before clinic phase but and before bacillary excretion, because in infected farms are present animals in different stages of evolution of the disease.

This study aimed to optimize the method for separating lymphocytes from the blood of cattle and sheep from the idea that, through assimilation, implementation and validation of new diagnostic methods

based on highlighting the cellular immune response and possible diagnostic correlations, we will be able to specify the diagnosis of subclinical paratuberculosis in as early a stage.

## 2.MATERIALS AND METHODS

The study started with the laboratory exploration on the blood collected on anticoagulant (heparin lithium) from cattle and sheep clinically healthy or diagnosed with paratuberculosis.

For an accurately assessment of a numbers of cells (lymphocytes) the variation is necessary to separate a homogeneous cell populations, of a high purity situation that will allow also their functional characterization.

To performe this, we used two techniques which allow the separation of different cell fractions from whole blood collected on anticoagulant by centrifugation in density gradient with adequate and compatible environment living systems: Ficoll and Percoll. Chemically speaking, Ficoll medium have in composition a branched carbohydrate whose density at 20 º C is 1.077 (1). Percoll medium is a biphasic medium containing in the liquid phase microbes embedded in silicon-coated polyvinyl pyrrolidone (PVP), which have a yellowish liquid, traslucid aspect.

Physical characteristics are: density (g / ml) 1.130 ± 0.005; conductivity max. 100, max.25 osmolality, viscosity 10 ± 5-20 ° C, p H 9.0 ± 0.5 at 20 ° C to 25 ° C (3).

**Principle of the method**: separation media alow to obtain a high purity of cell population starting from cell property gradually changing of the density in a continuous gradient separation (1).

**Working mode**: For this research, we used the products: Ficoll 1,077; Percoll in 1.5 M diluted with NaCl, with different ranges of density and taking into account the lymphocyte subpopulation.

Exploratory studies focused on: optimal centrifugation (1800 rpm, 2500 rpm, 2800 rpm)speed, the centrifugation temperature (range 4-25°C), duration of centrifugation (20, 30 minutes).

Preparation of Percoll solutions

Were achieved preparation of Percoll solutions of different densities, in accordance with the manufacturer by dilution in distilled water and sodium chloride solution. The used solutions were prepared immediately before use.

For dilution, was used 1.5 M NaCl solution and distilled water (Millipore) using the following procedure:

- we calculate the solution of diluted Percoll (depending on the number of samples taken in the study);

- was added a volume of Percoll undiluted, calculated using the following formula:

V = V0 [(d-0, 1) (d10-0, 9) / d0-1]

Where V0 is undiluted Percoll volume - in milliliters,

V- The final volume of solution of diluted Percoll

d- final solution density,

d10- NaCl, density -1.5 M

d0- undiluted Percoll density (1.130 g / ml);

-we add the distilled water to complete the final solution volume (2,3).

According to the data from scietific literature, the best way to separate the lymphocytes was using a medium density of 1.075 g / ml Percoll (1, 3).

Conditioning of a blood sample

-undiluted blood sample

-the blood sample (diluted) was combined in equal proportions with 1.5 M NaCl solution, PBS or cell culture medium RPMI 1640.

Transfer of the blood sample, undiluted and diluted

We put in contact the Ficoll solution with 1.077 density; Percoll solution with density 1.130 and 1.075 together with a blood sample. The test was performed in the following way: was transferred into a tube the desired amount of Ficoll, Percoll, after this we aspirated the undiluted or diluted blood by a pipette.

The tube with separation medium was blened with an angle of about 20 degrees to the horizontal and the blood from the pipette was deposited on the lower wall of the tube at a 2 cm distance from the edge of the Ficoll, Percoll. Evacuation of blood from the pipette need to done very slowly so that the contact between two fluids to be done very easily and the dispersion of blood in the contain of Ficoll, Percoll to be minimal.

After the blood evacuation into the tubes, we put the tubes in a vertical position so will remain to two completely separate liquid phase: lower phase consisting of Ficoll, Percoll and high blood phase (Fig.1-2)

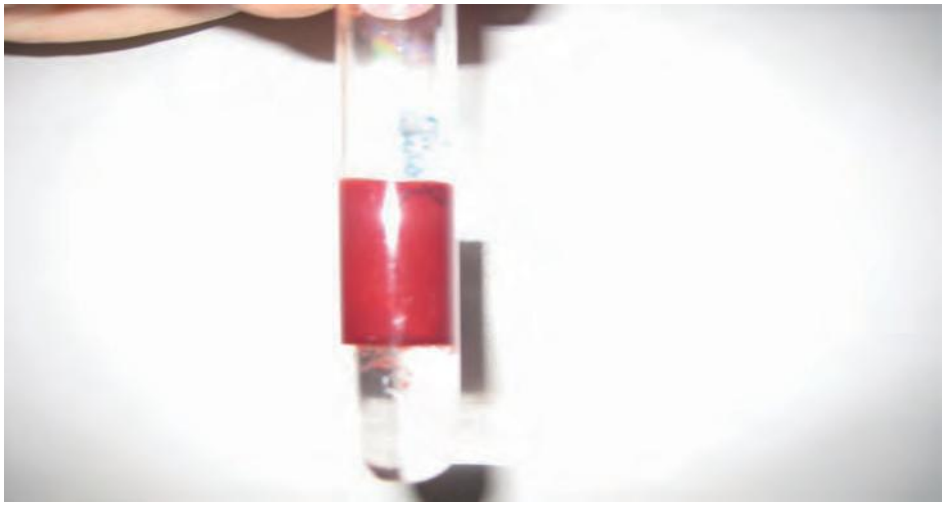

**Fig. 1. The blood sample (undiluted) before Ficoll gradient separation-1, 077**

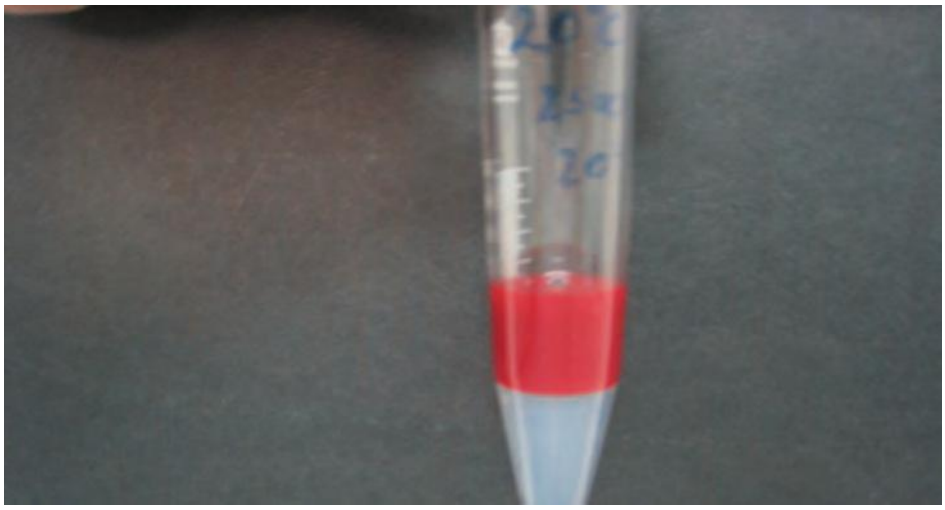

**Fig. 2. The blood sample (undiluted) before Percoll gradient separation – 1, 130**

The centrifugation of blood samples was permormed according to the following parameters:

- undiluted samples were centrifuged at 1800 rpm, 25 ° C - 20 minutes (Fig. 3, 4);
- ½ diluted samples with RPMI 1640 at 4°C-20-2500 rpm, 20°C-20-2500 rpm, 20°C-30-1800 rpm, 20°C-20-2800 rpm;
- ½ diluted samples with NaCl 1.5M, PBS(sterile PBS pH 7.2) were centrifuged at 4°C-20-2500 rpm, 20°C-30-1800 rpm.

## 2. RESULTS AND DISCUSSIONS

By bringing together the Ficoll solution of 1.077 density, Percoll solution of 1.130 density with an equal volume of blood collected on heparin lithium, centrifuged at 1800 rpm 25°C - 20 minutes, we saw the formation of two levels represented by red blood cells and plasma and also we observed the absence of lymphocytes band (Fig.3-4).

The separation of lymphocytes didn`t produced by dilution ½ of blood with RPMI 1640 medium and using the Ficoll separation and Percoll 1,077 and 1,130 media (Figure 5, 6).

For the blood samples diluted with 1.5 M NaCl solution or sterile phosphate buffer (PBS) pH 7.2 and mixed with an equal volume of Percoll density 1.075 we observed:

-the absence of lymphocytes band for the blood sample diluted with NaCl 1.5M and,

- the easily discernible lymphocyte band for blood sample diluted with PBS (fig.7-8).

To ensure a high sterile environment for cell separation, was used for dilution of the Percoll medium (1,130), 1.5M NaCl and distilled water filtered through a Millipore filter (0.45 μm).

Using the two components (filtered) to dilute the Percoll medium we notice the absence of lymphocytes band of both blood samples undiluted and diluted (Fig.9-10).

Spreading 3 ml of Percoll separation medium with 1, 075 density and then adding 3 ml of blood collected on heparin lithium and diluted ½ with RPMI 1640 medium, we observed the presence of red blood cells band, a band of lymphocyte between plasma and Percoll level (fig.11-12).

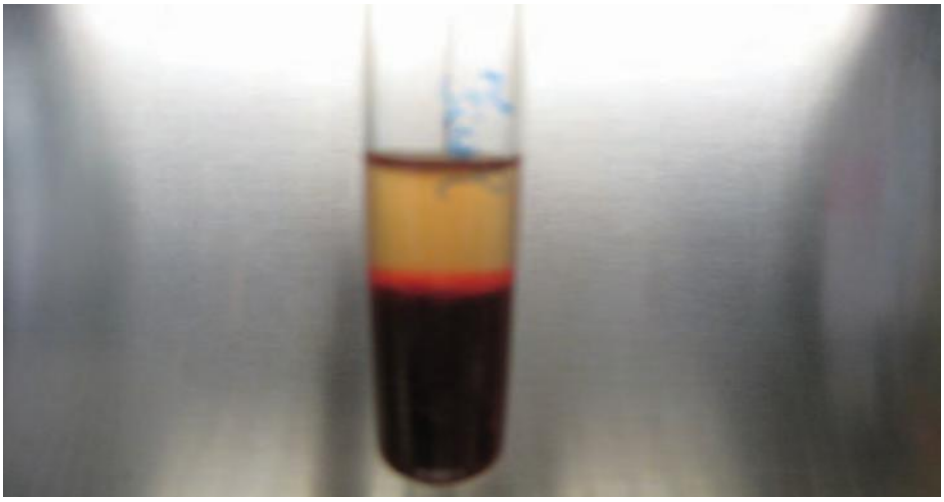

**Fig. 3. The blood sample (undiluted) after the Ficoll gradient separation 1.077 (25°C-1800rpm, 20 minutes)**

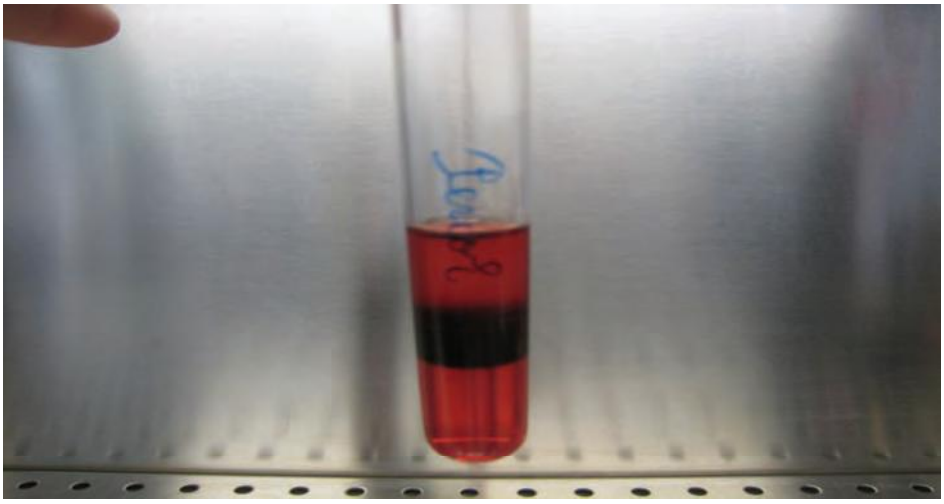

**Fig. 4.The blood sample (undiluted) after Percoll gradient separation 1.130 (25°C-1800rpm, 20 minutes)**

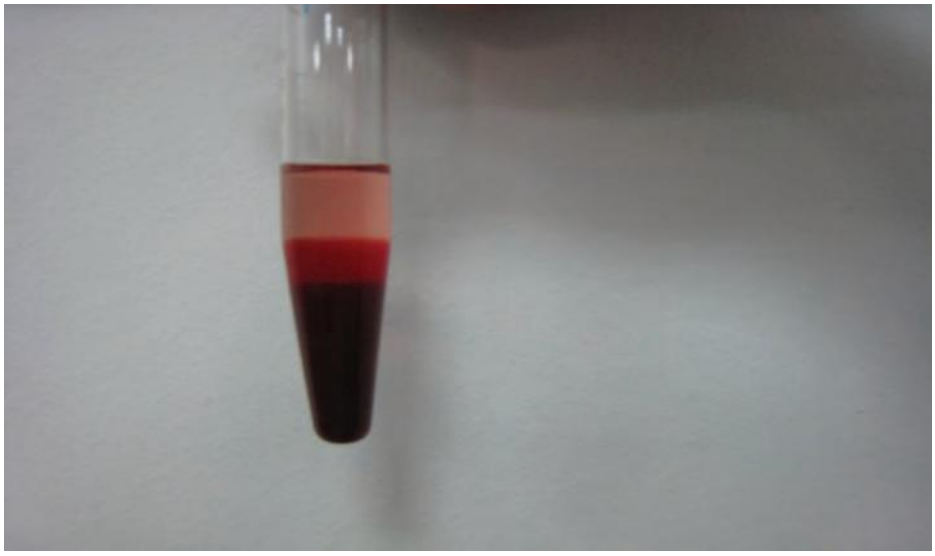

**Fig. 5. The blood sample (diluted with RPMI ½) after Ficoll-gradient separation 1.077 (4°C, 2500rpm, 20 minutes)**

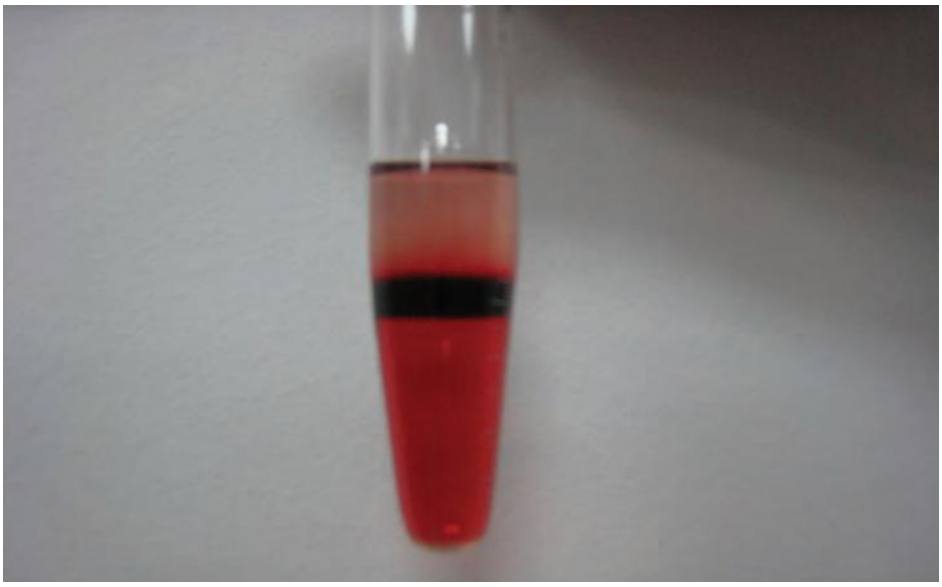

**Fig. 6. The blood sample (diluted ½ with RPMI) after Percoll gradient separation, 1.130 (20°C-2500rpm, 20 minutes)**

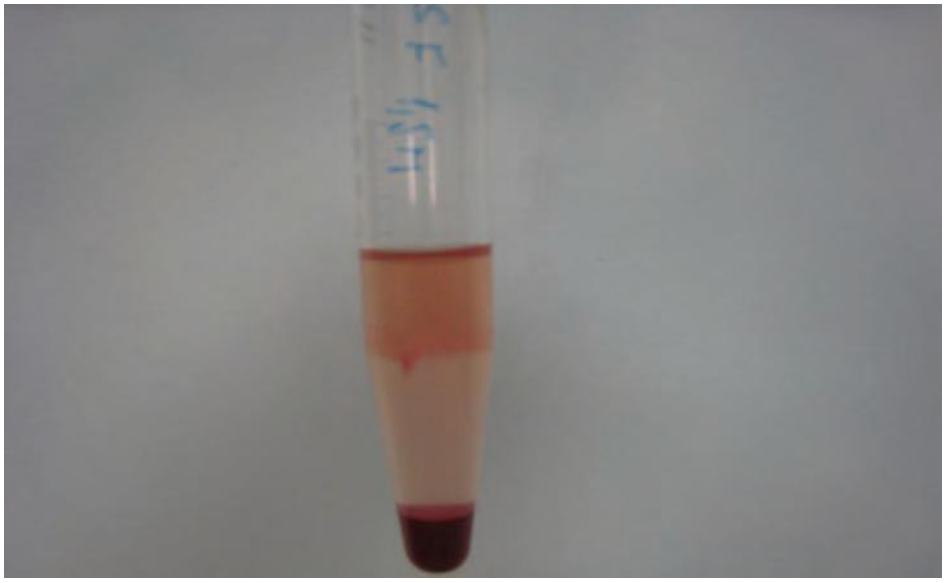

**Fig. 7. The blood sample (1.5 M SF dil ½) after Percoll gradient separation, 1.075 (4°C, 2500rpm, 20 minutes)**

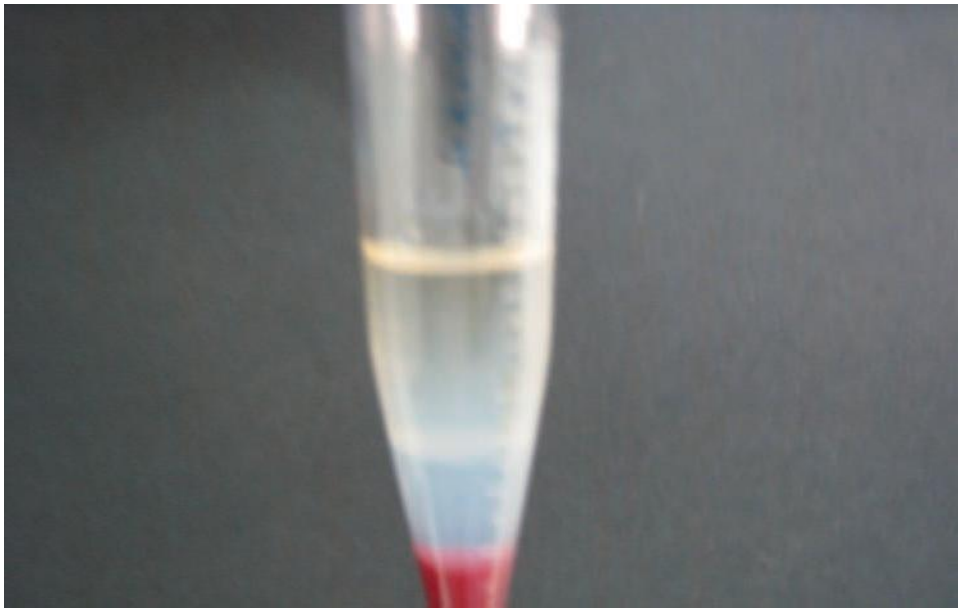

**Fig. 8. The blood sample (dil ½ with PBS) after Percoll gradient separation, 1.075 (4 °C, 2500rpm, 20 minutes)**

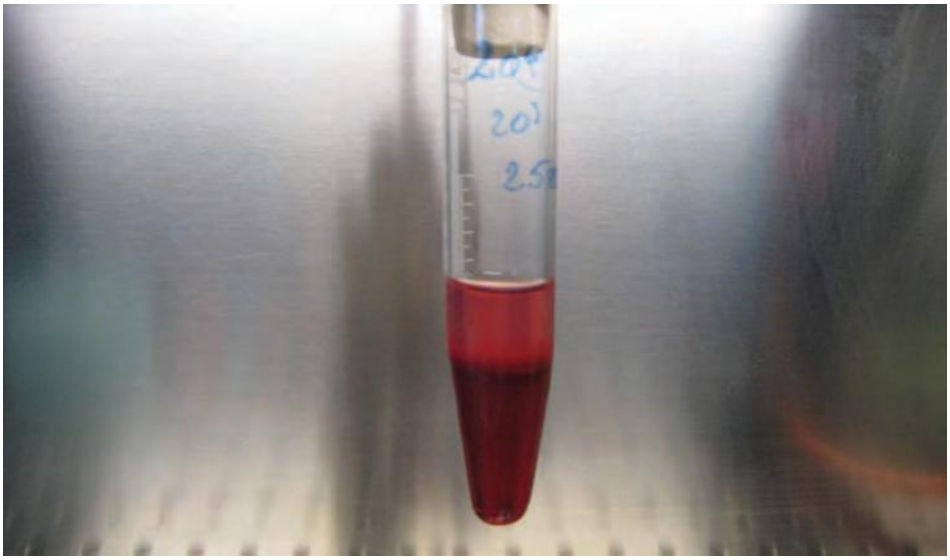

**Fig. 9 The blood sample (undiluted) after Percoll gradient separation in 1, 075 (sterile medium, distilled water filtered + filtered NaCl 1.5 M -20°C-2500rpm, 20 minutes)**

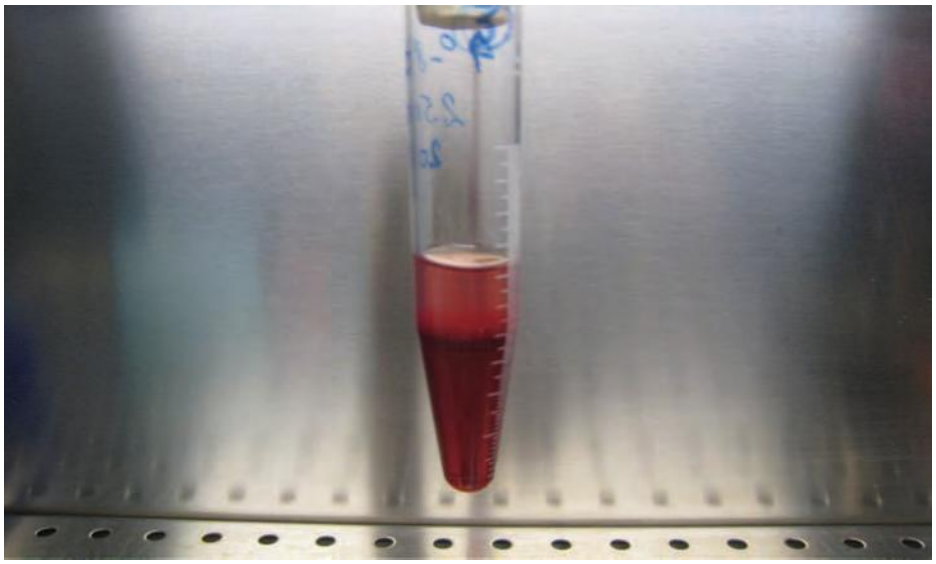

**Fig. 10 The blood sample (diluted 1 / 2 RPMI) after Percoll gradient separation in 1,075**
**(sterile medium, distilled water filtered + filtered NaCl 1.5 M -20°C-2500rpm, 20 minutes)**

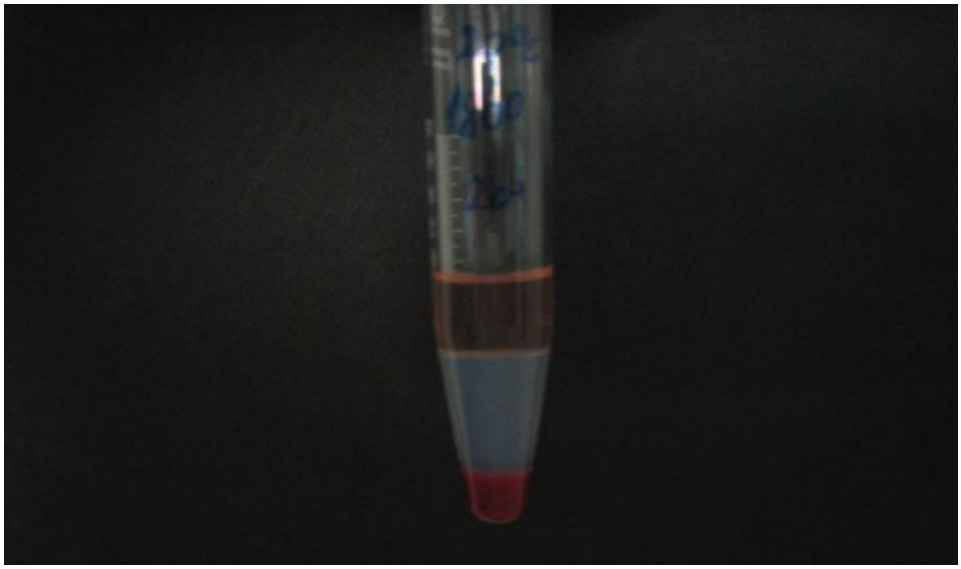

**Fig. 11. The blood sample (dil ½ with RPMI) after Percoll gradient separation in 1.075 (20°C-1800rpm, 20 minutes)**

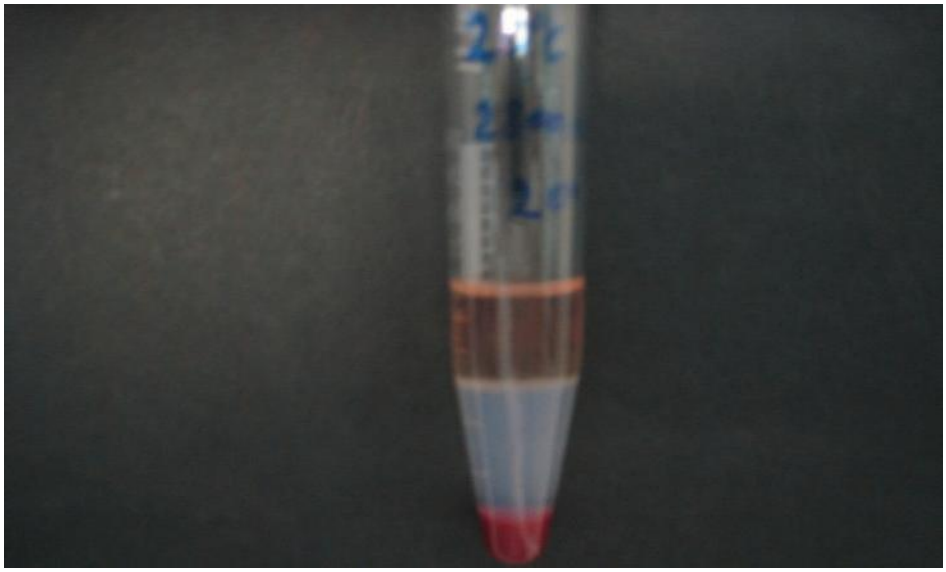

**Fig. 12.The blood sample (dil ½ with RPMI) after Percoll gradient separation in 1.075 (20°C-2800rpm, 20 minute)**

## 3. CONCLUSIONS

3.1 Separation of lymphocytes in medium Ficoll, Percoll with density gradient, using cattle and sheep blood can not be achieved without dilution of blood samples.

3.2 By using the Ficoll medium with density gradient separation - (1.077 density), can not be obtain the separation of lymphocytes from the cattle and sheep blood undiluted and diluted with RPMI ½.

3.3 By using blood ½ diluted with PBS (4 ° C, 2500rpm, 20 minutes, 20 ° C-1800rpm, 20 minutes) we obtain a better separation than using blood 1/2 diluted with 1.5 M NaCl (4 ° C 2500rpm, 20 minutes, 20 ° C-1800rpm, 20 minutes).

3.4 The best results were obtained by diluting blood with ½ rate of RPMI 1640 medium and by using the separation medium Percoll with density of 1.075 (20 ° C-1800rpm, 20 minutes, 20 ° C-2800 rpm, 20 minutes), using a 3 ml volume of of blood diluted ½ and by adding of a 3 ml Percoll 1, 075.

3.5 The purity of the cell mass separation is influenced by the centrifugation method for harvesting, the top level of the plasma must be removed properly and the suction operation of this level of cells should not include the lymphocytes level.

3.6 For obtaining of an optimal separation of lymphocytes it must be meet the following parameters:

- diluting the blood sample,
- sample dilution medium of blood sample,
- separation medium density,
- temperature and time of centrifugation,
- filtration of solutions for sterility reasons.

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# CONTRIBUTIONS TO PERIOCEUTIC THERAPY OF PERIODONAL DISEASE IN DOGS

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**Key words:** perioceutic, periodontal disease, dog

**SUMMARY**

In this study we tried to provide an alternative perioceutic treatment with long term efficacy for the periodontal disease in dogs. The association of human use products Perioflush and ChloSite with veterinary use products Stomorgyl and Germostop bucal is a viable protocol for the treatment of periodontal disease in dog. Perioceutic treatment with chlorhexidine base gel assures a fast healing of periodontal pocket and creates the assumption of a long term healing.

Mechanical debridment of calculus and plaque combined with local and systemic antimicrobial treatment have proved to be a viable solution in periodontal disease control.

This study presents an alternative therapy for periodontal disease in dogs using a combination of human use products and veterinary use products.

## 1. MATERIALS AND METHODS

The researches were made on 20 dogs, different breed, clinical cases with periodontal disease, presented in Surgery Clinics between 2008 and 2010. This dogs, nine males and 11 females, with ages between five and eleven years, were divided in two groups: control group and experimental group (table 1).

Both groups were first clinical and radiographical evaluated to assess the periodontal status. The clinical assessment was made following periodontal probing and sulcus bleeding index, calculus index and periodontal pocket depth interpretation (tables 2, 3, 4). Under general anesthesia (acepromazine 1%, ketamine 10% and Propofol 1%) mechanical scaling was performed on all dogs. Afterwards, each group was differently approached.

On control group, after mechanical debridement, the periodontal pocket was flushed with chlorhexidine solution , synulox (amoxiciline

and clavulanic acid) was recommended as systemic antimicrobial therapy and Germostop bucal (chlorhexidine), twice daily, as topical antimicrobial.

***Table 1***

**Dogs distribution in groups**

| Group | Nr. | BREED | AGE | SEX |
|---|---|---|---|---|
| CONTROL | 1 | Common | 8 | Male |
| | 2 | Common | 7 | Male |
| | 3 | Peckinez | 9 | Female |
| | 4 | Shi-Tzu | 6 | Male |
| | 5 | Common | 10 | Female |
| | 6 | Peckinez | 8 | Female |
| | 7 | Teckel | 8 | Male |
| | 8 | Common | 5 | Female |
| | 9 | Pudell | 9 | Female |
| | 10 | Common | 6 | Female |
| XPERIMENTAL | 1 | Pekinez | 7 | Female |
| | 2 | Common | 9 | Female |
| | 3 | Peckinez | 9 | Male |
| | 4 | Baset | 6 | Male |
| | 5 | Shi-Tzu | 8 | Female |
| | 6 | Common | 11 | Male |
| | 7 | Common | 9 | Male |
| | 8 | Common | 7 | Female |
| | 9 | Pudell | 7 | Male |
| | 10 | Common | 10 | Female |

***Table 2***

**Sulcus bleeding index (SBI)**
**(Wiggs R.B. and Lobprise Heidi, 1997)**

| Value | Description |
|---|---|
| 0 | Healthy appearance, no bleeding on sulcus probing |
| 1 | Apparently healthy, showing no change in color or swelling, but slight bleeding from sulcus on probing |
| 2 | Bleeding on probing, changing of color due to inflammation, no swelling or macroscopic edema |
| 3 | Bleeding on probing, change in color, slight edematous swelling |
| 4 | Bleeding on probing, change in color, obvious swelling |
| 5 | Bleeding on probing, spontaneous bleeding, change in color, marked swelling with or without ulceration |

*Table 3*

**Calculus index (CI)**
**(Wiggs R.B. and Lobprise Heidi, 1997)**

| Value | Description |
|---|---|
| 0 | No calculus |
| 1 | Supragingival calculus extending only slight below the free gingival margin |
| 2 | Moderate amount of supragingival and subgingival calculus, or subgingival calculus only |
| 3 | Abundance of supragingival or subgingival calculus |

*Table 4*

**Periodontal pocket depth interpretation (Wiggs R.B. and Lobprise Heidi, 1997)**

| Depth | Interpretation |
|---|---|
| < 4 mm | Healthy |
| > 4 mm | Satisfactory |
| > 6 mm | Diseased periodontum |

On experimental group, after mechanical debridement, the periodontal pocket was flushed with Perioflush and afterwards ChloSite gel was introduced in it. For systemic antimicrobial therapy was used Stomorgyl (spiramicine and metronidazol) and as local antimicrobial was used Germostop bucal.

Both, Perioflush liquid and ChloSite are human use products. Perioflush liquid (Dental Life Sciences, UK) is a 2 ml syringe with applicator that contains hydrogen peroxide, chlorhexidine and phosphoric acid. ChloSite (Ghimas, Bologna, Italia) is a plastic gel that solidifies in periodontal pockest presented as one-dose syringe and contains chlorhexidine digluconate and chlorhexidine dihidrochloride in 1:2 rapport.

The clinical and radiographical examination was made in three different moments: after mechanical debridement, at one month and at three months.

## 2. RESULTS AND DISCUSSIONS

From the results obtained initially at clinical evaluation we can observe the uniformity of the individuals of both groups concerning periodontal status (tables 5 and 6).

The values of calculus index noted at one month revealed the absence of calculus and plaque, or its presence in small amounts. We considered that these aspects are due to the short time interval between scaling and evaluation and due to the prophylaxis with Germostop bucal. At three months evaluation we have not observed more abundant deposals on teeth surfaces. The evaluation of calculus index is subjective, being correlated with individual predisposition, diet and home care. The assessment of calculus index was made to determine the tendency of periodontal disease to reemerge based on the mechanical, irritant ground provided by calculus.

***Table 5***

**Sulcus bleeding index values on both groups in the moment of presentation and at one and three months interval**

| Group | Dog no. | 0 | 1 month | 3 months |
|---|---|---|---|---|
| CONTROL | 1 | 3 | 0 | 1 |
| | 2 | 4 | 1 | 1 |
| | 3 | 3 | 0 | 0 |
| | 4 | 3 | 0 | 1 |
| | 5 | 4 | 1 | 1 |
| | 6 | 3 | 1 | 1 |
| | 7 | 5 | 2 | 2 |
| | 8 | 3 | 0 | 0 |
| | 9 | 4 | 1 | 1 |
| | 10 | 3 | 1 | 1 |
| EXPERIMENTAL | 1 | 4 | 0 | 0 |
| | 2 | 4 | 0 | 1 |
| | 3 | 3 | 1 | 1 |
| | 4 | 4 | 0 | 0 |
| | 5 | 3 | 0 | 0 |
| | 6 | 5 | 1 | 1 |
| | 7 | 4 | 0 | 0 |
| | 8 | 3 | 0 | 0 |
| | 9 | 3 | 0 | 0 |
| | 10 | 4 | 1 | 1 |

The sulcus bleeding index, periodontal pocket depth and radiographical aspects have a higher objective value being more useful in periodontal status assessment.

The values of sulcus bleeding index were between three and five in the moment of first presentation, correlated with clinical aspects. We have observed bleeding on probing, changes in gingival color and gingival edema. At one month evaluation, the values of this index are

between zero and one (with one exception – dog no. seven from control group). Nevertheless, a slight difference can be observed between the two groups concerning the predominance of the value zero at the experimental group. At three months these values are maintained at the experimental group but on some dogs from control group we have observed a deterioration of the periodontal status with bleeding on probing.

***Table 6***

**Periodontal pocket depths (in millimeters) on both groups in the moment of presentation and at one and three months interval**

| Group | Dog no. | 0 | 1 month | 3 months |
|---|---|---|---|---|
| CONTROL | 1 | 6 | 5 | 5 |
| | 2 | 7 | 5 | 4 |
| | 3 | 6 | 4 | 4 |
| | 4 | 8 | 6 | 6 |
| | 5 | 8 | 7 | 6 |
| | 6 | 6 | 5 | 5 |
| | 7 | 9 | 7 | 7 |
| | 8 | 7 | 6 | 6 |
| | 9 | 8 | 6 | 7 |
| | 10 | 7 | 6 | 5 |
| EXPERIMENTAL | 1 | 6 | 4 | 3 |
| | 2 | 7 | 4 | 4 |
| | 3 | 6 | 5 | 3 |
| | 4 | 9 | 6 | 4 |
| | 5 | 7 | 5 | 4 |
| | 6 | 8 | 5 | 3 |
| | 7 | 7 | 4 | 3 |
| | 8 | 6 | 4 | 3 |
| | 9 | 6 | 4 | 4 |
| | 10 | 8 | 5 | 3 |

The periodontal pocket depths were first between six and nine milimeters. At one month, on control group the depths were between four and seven milimeters and at experimental group were between four and five with one exception (dog no. 4 that had a 6 mm pocket depth, initially 9 millimeters).Clear differences between groups were observed at three months when on control group we obtained similar values as at one month and we even observed a slight worsening of those. In contrast, on experimental group the values showed an improvement which means cessation of the destructive process and beginning of the reparative process.

The radiographical aspects first obtained revealed the presence of infrabony pockets especially on distal root, with vertical bone resorption. These aspects are positivelly correlated with the values of sulcus bleeding index and periodontal pocket depth. Radiographical examination made at one month did not showed major differences and the lack of correlation with the values of pocket depths in some cases being due to the formation of conjunctive tissue that is not mineralized. At three months, the radiographical density of new bone from the pockets is higher which shows the presence of alveolar bone reparative process (more obvious in the experimental group). This positive effect of chlorhexidine base products on periodontal pockets is also described by other authors (Robinson J.G.A., 1995, Tepe J.H., 1983, Yue *et al*., 2004).

## 3. CONCLUSIONS

3.1. Mechanical debridement associated with systemic treatment with Synulox and topical application of Germostop bucal is followed only by a passing improvement of periodontal status, being reserved on long term.

3.2. Perioceutic treatment with chlorhexidine base gel assure a fast healing of periodontal pocket and create the assumption of a long term healing.

3.3. The association of human use products Perioflush and ChloSite with veterinary use products Stomorgyl and Germostop bucal is a viable protocol for the treatment of periodontal disease in dog.

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# EMPHASIZE THE LEVEL OF CONTAMINATION AND MICROBIAL BIOFILM ON INERT SURFACES FROM MEAT AND MILK PLANT

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**Key words:** Microbial biofilm, processing plant, surfaces

**SUMMARY**

The aim of this study was to emphasize the level of contamination and presence of microbial biofilms on various surfaces in food processing plants. The research was conducted in three processing units: a milk processing unit (LP), a processing unit of pork (PC 1), a unit of slaughter-processing of pork (PC 2). Assess the level of contamination, of inert surfaces with different degrees of finish, was done by direct counting plate and evidence the presence of microbial biofilm, by confocal microscopy, after collection of samples by scraping and staining with acridine orange, was made. Microbial biofilm was detected in five areas of the milk processing unit (60% of the total area examined) and two areas of meat processing unit PC 1. The slaughter-processing unit (PC 2) reveals the presence of microbial biofilm on nine areas (52.2%) of all areas examined. Areas on which the presence of microbial biofilms was identify were areas with different levels of roughness: low-grade stainless steel finishes and corrosion zones, high-porosity plastic, rubber and epoxy resin floor rough.

The biofilm is a community of microorganisms enhanced in an extra cellular substance, such as polymer, attached to a surface. In this form bacteria can be found in nature, in body and in various systems designed by humans (5).

The biofilm is frequently encountered on surfaces that come into contact with foodstuffs, and in certain circumstances may constitute a reservoir of potentially pathogenic bacteria to the consumer. Equipment used in food processing area, especially those improperly cleaned, can become sources of microbial contamination. Even with the application of good hygiene practices, microorganisms can remain on the surface of the equipment and form biofilm. More difficult to clean areas (joints, rubber seals), wet surfaces (flooring, conveyor belt), surfaces that may corrode the aging structures are susceptible to biofilm formation (1,5). Transmission of microorganisms to finished products can be achieved either through direct contact with surfaces contaminated equipment or by aeromicroflora, resulting from the washing of equipment coming into contact with food.

The aim of this study was to emphasize the level of contamination and presence of microbial biofilms on various surfaces in food processing plants.

## 1. MATERIAL AND METHODS

For research were chosen four processing plant: a milk processing unit (LP), a processing unit of pork (PC 1), a unit of slaughter-processing of pork (PC 2).

Establishing the level of contamination and evidence the presence of biofilms was performed on inert surfaces with different degrees of finishing. Surfaces consisting of different materials (steel, PVC, glass, plastic, etc.) with different degrees of finishing (finished surfaces and joints of the pipes) were chosen

Assessing the level of contamination of inert surfaces was done by direct counting plate. Biofilm structure was identified using various methods highlighted by fluorescent dye substances substrate (fluorescence microscopy and confocal microscopy).

Sanitation samples were collected from the equipment, tools, and construction elements surface, which may come into contact with raw material, with half-finished or finished products (flooring, wall cladding panels, tiles, etc.). Sampling (processed after the method) was performed using sterile swabs, from an area of 100 $cm^2$ or 25 $cm^2$, depending on the test used.

Sanitation samples were transported in the laboratory under refrigeration. Setting the level of contamination was done by assessing the following areas of microbiological indicators: total number of aerobic germs, the number of *Enterobacteriaceae* and the number of bacteria of the genus *Pseudomona*s, according to standards.

To emphasize the presence of microbial biofilm sampling method by scraping with a scalpel was used, in the proximity of areas where sanitation samples were taken. Each sample was taken in duplicate on each glass slide, which was previously cleaned and degreased with alcohol 70%. For each sample, the scalpel was sterilized in advance by flaming. In the laboratory, samples were submitted to fixation and staining. The first set of samples was stained with acridine orange (AO), as described by Hoff et al. (1984). The samples were fixed on the slide with ethanol 96% for two minutes. Slide staining was performed for one minute, and excess dye was removed by successive washing with distilled water. Slides so prepared were dried at room temperature.

Examination of slides was done using epifluorescence microscope Leica model DM 2500, having as a source of UV mercury vapor lamp. To achieve wavelength capable of producing excitation fluorochrome (acridine orange), we used an excitation filter BP 450-490 nm. Examination of samples was done with immersion objectives 63x, respectively, 100x, using an immersion liquid with refractive index 1.518. The microscope investigations were made with a digital camera Leica DFC 350 FX monochrome model with a resolution of 1.4 megapixels and FireWire. The images were stored and processed using LAS AF program (Leica Application Suite Advanced Fluorescence).

A copy of each sample was stained with acridine orange and second, by the *Gram* method.

## 2. RESULTS AND DISCUSSION

In samples taken from areas of the milk processing unit and a meat processing plant was found the presence of microorganisms as isolated colonies or included in the matrix of the biofilm.

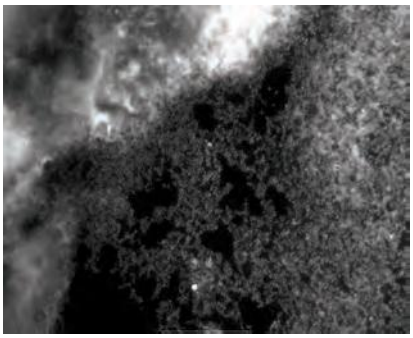

**Fig. 1. Microbial biofilm from the inner surface of the milk cooling tank. Col. acridine orange fluorescent microscopy examination 63x objective**

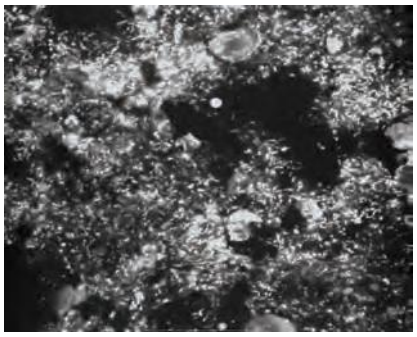

**Fig. 2. Microbial biofilm on the inner surface of the harvested fat separator. Col. acridine orange fluorescent microscopy examination 100x objective**

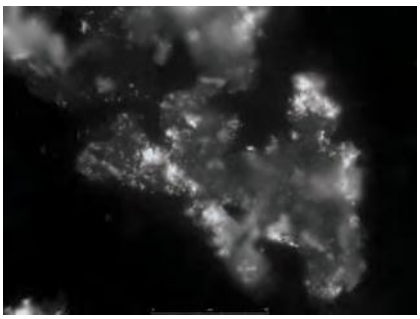

**Fig. 3. Harvested microbial biofilm on the surface of the stirring blades milk cooling tank. Col. acridine orange fluorescent microscopy examination 63x objective**

In the milk processing plant (PL) clumps of microorganisms included in the biofilm were found in the following collection points: the inner surface of the cooling tank, the inner surface of the cream separator, the floor surface, the inner surface of the pipe before entering pump milk and the rubber gasket surface of the blade cooling tank (Figures 1-3).

Sanitation samples collected from the same point has been highlighted the presence of microbial biofilms, the flow of milk processing, the level of contamination (assessed by inoculation on culture media), was different. Thus, less accessible areas to be cleaned, respectively: the inner surface of the pipe before entering pump milk from the rubber gasket, the inner surface of the cream separator and the floor surface, the overall level of contamination ranged from $1x10^4$ and $5x10^5$, with a number of enterobacteria $5x10^2$ - $5x10^3$ and 80 bacteria/$cm^2$ of the genus *Pseudomonas*.

Czechowski et al. cit. by Amy Lee Wong (2004) found the presence of biofilm in the rubber lining equipment in milk processing units. In the other samples (area of the agitator blade cooling tank and the inner surface of the stainless steel cooling tank and storage), general level of contamination ranged from $9x10^2$ to $1x10^4$, the enterobacteria from and the bacteria from *Pseudomonas* genus were not emphasized .

Overall, milk processing unit, the presence of microbial biofilm was found on surfaces that had a certain degree of roughness: the surface of the rubber gasket before entering pump milk, milk from the tank paddle mixer, cream separator and the floor surface. Most surfaces in dairy are stainless steel, with a high degree of finishing, where microorganisms adhere more difficult, that explains why identifying the presence of microbial biofilm on the surface of several objectives was done.

The slide *Gram* stained examination evidence the presence of microbial biofilms (the surfaces that come into contact with milk before pasteurization), in most cases (except for milk cooling tank area after pasteurization) were predominant *Gram*-positive cocci and *Gram* negative bacilli. According to our finding *Amy Lee Wong* (2004) frequently isolated *Gram*-positive cocci and the area of milk processing equipment.

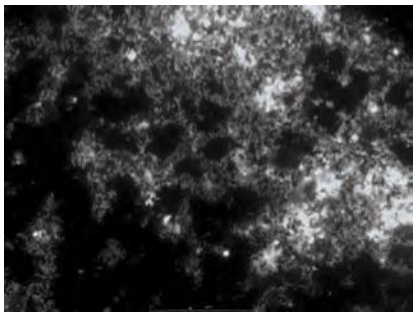

**Fig. 4. Microbial biofilm on inner surface sterilizer for knives. Col. acridine orange fluorescence microscope examination, objective 63**

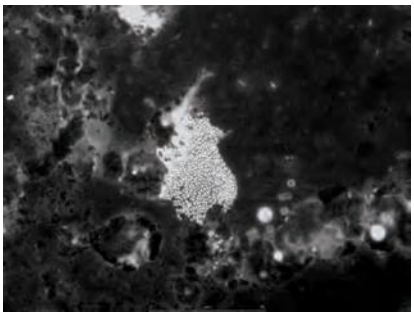

**Fig. 5. Microbial biofilm on the surface of rubber apron. Col. acridine orange exam. fluorescence microscope, 63x objective**

The surfaces were the microbial biofilm were isolated especially those are in contact with milk can become sources of contamination with microorganisms that can detach periodically from these structures and contaminate food.

In meat processing unit (overall microbial load showed varying levels, from 4 x $10^2$ on the inner surface sterilizer cfu/cm$^2$ 1.7 x$10^4$ cfu/cm$^2$ sterilizer lid surface and rubber apron. Enterobacteria contamination of these areas varied from 1: 2.1 x $10^3$, and the germs of the genus *Pseudomonas* were present in small numbers 1 to 12 cfu/cm$^2$.

The presence of microbial biofilms could only emphasize in the following areas: the inner surface and cover (plastic) knife sterilizer and butchers apron rubber surface. On these surfaces microorganisms were present in the form of clusters included in the biofilm structure type. Moreover, evidence of biofilms on the surfaces of the meat processing industry has been reported in other literature. On the surface transporter ribbon, cutting tables, knives, saw the microbial biofilm formation have been identified (1,5).

Although samples were taken and on other surfaces, which, theoretically, should have identified the presence of microbial biofilms,

however, has not been revealed. Perhaps the failure is due to sampling method, the scraping of the biofilm. For example, *Holah et al.* cit by Zottola, 1995, believes that by removing the collection of samples on surfaces is more accurate if the level of contamination exceeds $10^5/cm^2$ and that under direct microscopic examination of these values would be more useful.

The slide stained by *Gram* method examination, evidence the presence of *Gram* negative bacilli *Gram*-positive cocci were predominant.

The processing unit of pork slaughter PC 2 has revealed the presence of microbial biofilms on surfaces made of different materials (steel, plastic and epoxy resins) by fluorescence microscopy. We identified the presence of microbial biofilms in a number of 13 points on the meat processing line.

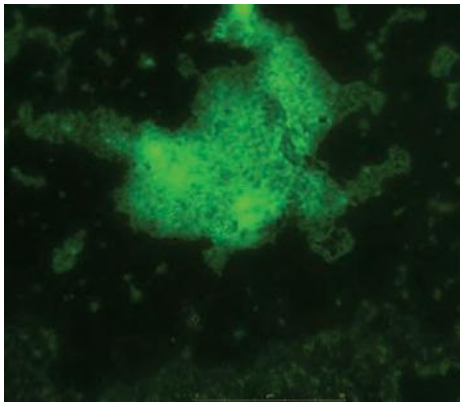

**Fig. 6. Microbial biofilm on the surface of the shuttle collected plastic. Col. acridine orange fluorescent microscopy examination 63x lens inside**

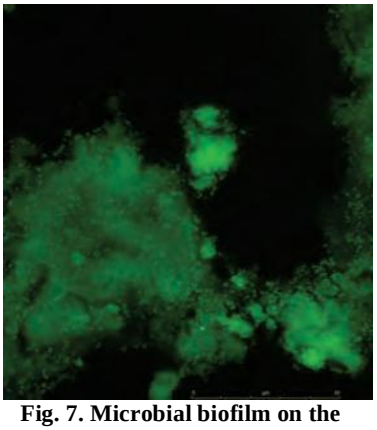

**Fig. 7. Microbial biofilm on the surface of stainless steel trolley. Col. acridine orange fluorescent microscopy examination 63x objective**

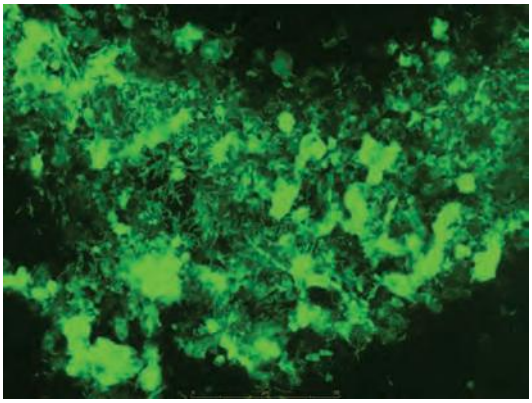

**Fig. 8. Microbial biofilm on the surface groove. Col. acridine orange fluorescent microscopy examination 63x objective**

In samples collected from cutting room reveal the presence of biofilm on surfaces of stainless steel (stainless steel work table, transport carts inside, knife cutting, hanging), plastic (cake cutting, turnip, PVC wall , USA) and epoxy (floor) (figures 6-8). In samples collected from these objectives sanitation overall level of contamination was $10^2$ - $10^4$ cfu/cm$^2$ without intestinal bacteria or germs such as *Pseudomonas*, are present in significant numbers in these areas. Identification of biofilm on stainless steel surfaces was usually in the joints to finish the degree was lower with increased possibilities of bacterial biofilm formation, which favors the degradation of stainless steel for corrosion and loss of the original traits (smooth surface) at this level. Plastic surfaces and epoxy resin floor surface, the possibility of biofilm formation is much higher due to the high degree of roughness of these surfaces, the possibility that bacterial film accession and retention at this level and as a result of the training areas Plastic cracks appeared in their aging. The slide stained by *Gram* method examination, evidence the presence of microbial biofilms, *Gram* negative bacilli were and *Gram*-positive cocci.

Other researchers have shown the presence of microbial biofilms on food surfaces. For example, *Mamadhavi Manijeh and col.* (2008) revealed *Salmonella enteritidis's* ability to form biofilm on stainless steel surfaces. The results are just the first step in our attempt to identify and characterize the biofilm to find the best ways to prevent removal and training.

## 3. CONCLUSIONS

3. 1. The presence of microbial biofilm was emphasize on five areas of the milk processing unit (60% of the total area examined) and two areas of meat processing unit PC 1.

3. 2. The slaughter-processing unit (PC 2) the presence of microbial biofilm on nine areas (52.2% of total area examined) was emphasize.

3. 3. Areas on which to identify the presence of microbial biofilms were areas with different levels of roughness: low-grade stainless steel finishes and corrosion zones, high-porosity plastic, rubber and epoxy resin floor rough.


## ACKNOWLEDGEMENTS

This study was supported by the project no. 1101 from 2009**,** COD 902 financed by Ministry of Education, Research and Innovation – National University Research Council We also thank the staff of the processing plant and retail unit involved in this study for facilitating access.


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# MICROBIAL BIOFILM EMPHASIS ON CARCASSES SURFACE

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Key words: Microbial biofilm, carcasses, ATP

## SUMMARY

Purpose of this study was to determine the level of contamination and emphasize, by fluorescence microscopy, the presence of microbial biofilm on the surface of bovine, pigs and poultry carcasses. The research was conducted in a meat processing plant and a retail unit. To assess the level of contamination of carcasses the NTGMA, number of Enterobacteriaceae and the number of bacteria of the genus *Pseudomonas* were determined. The presence of microbial biofilm after sampling by scraping and staining by confocal microscopy was made. To determine the level of ATP on the surface of the meat two instruments Hy-Lite 2 and PD Lumitester 10N were used. Microbial biofilm was emphasis on the surface of cattle carcasses (42% of samples) and pork carcasses (29.4% of samples). The overall level of contamination in areas where it emphasized the presence of microbial biofilms, has fluctuated on average from $10^2$-$10^4$ cfu/cm$^2$.

Quality and safety of the raw material used in the meat industry influence the quality of finished product and also the preservation of products. The level of contamination of carcasses at the end of the technological process is influenced by the existing sanitary conditions along the flow of slaughter (8). Largest source of microorganisms is the intestinal contents of slaughtered animals, through whose carcasses may become contaminated with pathogenic micro-organisms to the consumer (E. coli, salmonella, etc.). By using classical methods sometimes the results are obtained as the carcasses were processed and delivered, it is therefore necessary to use rapid methods of identification of possible contaminated areas on the surface of carcasses. A food can be colonized by organisms form microbial biofilms, which may represent, in certain circumstances, a potential hazard to human consumers (3). There are relatively few investigations that have revealed the presence of microorganisms on the surface of food and even less on the surface of carcasses (6,7,).

Purpose of this study was to determine the level of contamination and to emphasize the presence of microbial biofilm by fluorescence microscopy on the surface of pig, beef and poultry carcasses.

## 1. MATERIAL AND METHOD

Two units for research were chosen: a pork meat processing unit (PC 1) and a retail unit (VA).

To assess the level of contamination direct counting plate was done. Biofilm structure was identified using various methods highlighted by fluorescent dye substances substrate (fluorescence microscopy and confocal microscopy).

Samples were collected from the surface of carcasses of pigs, cattle and poultry. Samples (prepared by standardized method) were performed using sterile swabs, from an area of 100 $cm^2$ or 25 $cm^2$, depending on the test used. Samples were transported to the laboratory under refrigeration. The level of contamination by assessing the following of microbiological indicators: total number of germs, the number of Enterobacteriaceae and the number of bacteria of the genus *Pseudomonas* according to standards, was done.

To emphasize the presence of microbial biofilm sampling method by scraping with a scalpel was used, in the proximity of areas where sanitation samples were taken. Each sample was taken in duplicate on each glass slide, which was previously cleaned and degreased with alcohol 70%. For each sample, the scalpel was sterilized in advance by flaming. In the laboratory, samples were submitted to fixation and staining. The first set of samples was stained with acridine orange (AO), as described by Hoff et al. (1984). The samples were fixed on the slide with ethanol 96% for two minutes. Slide staining was performed for one minute, and excess dye was removed by successive washing with distilled water. Slides so prepared were dried at room temperature.

Examination of slides was done using epifluorescence microscope Leica model DM 2500, having as a source of UV mercury vapor lamp. To achieve wavelength capable of producing excitation fluorochrome (acridine orange), we used an excitation filter BP 450-490 nm. Examination of samples was done with immersion objectives 63x, respectively, 100x, using an immersion liquid with refractive index 1.518. The microscope investigation were made with a digital camera Leica DFC 350 FX monochrome model with a resolution of 1.4 megapixels and FireWire. The images were stored and processed using LAS AF program (Leica Application Suite Advanced Fluorescence).

A copy of each sample was stained with acridine orange and second, by the Gram method.

To determine the level of ATP on the surface of the meat they use special putty and light intensity from the resulting reaction was read at the two instruments Hy-Lite 2 and PD Lumitester 10N.

## 2. RESULTS AND DISCUTIONS

The overall level of contamination of carcasses at a reception in the PC unit was significantly increased compared to the normal level of surface contamination. Thus, in four of the eight regions on the surface of various carcasses, general contamination exceeded the maximum of 4 log cfu/cm$^2$ established by EU Regulation 1441/2007. Those regions where: neck and chest (4.37 log cfu/cm$^2$), front of thigh (4.49 log cfu/cm$^2$) lateral abdominal region (5.45 log cfu / cm$^2$) and coccygeal region (4.55 log cfu/cm$^2$). In only two samples for Enterobacteriaceae (front of thigh and cocccigian region), their number was higher than the limit, 2 log cfu / cm $^2$, the rest of values were below this value.

The presence of microorganisms of the genus *Pseudomonas* on the carcasses surface was found in six of the eight regions in which they have been sampled. The high level of surface contamination of carcasses found in this plant is the result of contamination during transportation or multiplication of microorganisms at low temperatures due to refrigerated storage of carcasses in a long time. Fact is that the manufacture a raw materials with a high level of contamination that leads to highly contaminated products that require.

Regarding ATP determination on the surface of carcasses by HyLite method an increased of the amount of ATP in six of the eight areas have been found. The values were varied from 213 URL/cm$^2$ from thoracic regions, to 2340URL/cm$^2$ lateral abdominal regions. Only two regions values obtained were below the limit of 500 URL / 25cm2 (thoracic region and cervical region).

By Lumitester method, a greater variation in the amount of ATP in samples collected from different regions of the carcass, were obtained. Thus, the amount of ATP ranged from 16 URL/cm$^2$ (thoracic region) to 13,500 URL/cm$^2$ (neck and chest). In six of the eight regions examined the results were below 200 URL/cm$^2$.

One of the limits of the apparatus used in the experiments is that does not distinguish between the ATP from microbial and no microbial sources. In fact on the surface of carcasses is a large amount of ATP from other sources. In addition, meat microflora on the surface (different for carcasses of beef and pork or chicken) is represented by a heterogeneous population at different stages of growth and different content of ATP (2,5).

The correlation coefficient obtained between the classical and the method was 0.08 Hylite and Lumitester method was 0.55, which

indicates the existence of a weak positive correlation with the reference method.

On the surface of beef carcasses from retail unit (VA 1) microbial level of contamination fluctuated from 2.2 log cfu/$cm^2$ samples collected from coastal area to 4.36 log cfu/$cm^2$ from the region. thoracicoabdominal In three of the seven samples collected the number of germ exceeded 3.4 log cfu/$cm^2$ (3.42 log cfu/$cm^2$ pulp region, the thoracoabdominal region 4.36 log cfu/$cm^2$ and chest 3.55 log cfu / $cm^2$). Intestinal bacteria were present in the three objectives of the harvest: pulp region, the region thoracoabdominal external and internal. Although there are no legislative limits on microbial load of carcasses into retail units, however, exceed the values obtained for the number of germs on the surface of beef carcasses at the slaughterhouse, 3.5 log for NTGMA cfu/$cm^2$ and 1.2 log cfu/$cm^2$ for enterobacteria. The processing of such carcasses in the meat products or cutting into pieces and will decrease the preservation of final products.

Regarding the amount of ATP on the surface of beef carcasses in the samples examined by HyLite method in all cases were obtained values below 500 25URL/ $cm^2$.By Lumitester method in only two regions, the internal face and neck muscle thoracoabdominal, values exceeded 200 URL / 25 $cm^2$. The correlation coefficient with the standardized methods was 0.14 and 0.11for Lumitester and HyLite method.

In contradiction with our results, Bautista et al. 2007 obtained a positive correlation with the classical method in all the 159 samples collected from the surface of beef carcasses.

Chicken carcasses from retail unit (VA 1) showed a contamination level lower than that found for beef carcasses. Thus, N.T.G.M.A. ranged from 177 cfu/$cm^2$ axillaries region, at 795 cfu/$cm^2$ in the chest region. Intestinal bacteria were present in small numbers on all surfaces examined. In the chest, the overall load and the number of enterobacteria were raised to reveal the presence pseudomonadelor. The device HyLite ATP values were relatively low (below 20 URL/$cm^2$) and Lumitester method were on average 3000URL/$cm^2$. The correlation coefficient between the classical and the HyLite method was 0.11 and Lumitester method was 0.68, average positive correlation considered.

A food can be colonized by microbial biofilms, which may represent, in certain circumstances, a potential hazard to human consumers. There are relatively few investigations that have revealed the presence of microorganisms on the surface of food and even less on the surface of carcasses (3).

Analysis of images obtained from samples taken from surface of pigs, cattle and poultry carcasses was found the presence of microorganisms as isolated colonies or included in the matrix of the biofilm.

On the surface of pork meat carcasses from processing plant (PC 1) clumps of microorganisms included in the biofilm were found in the sternal region and the coast region and the cut surface of meat pieces, with at least 5 days aging on the Internal pulp, coccygeal region and the surface of chop muscle

The level of contamination, in areas where the presence of microbial biofilm was emphasized, fluctuated from $10^2$-$10^4$ cfu/cm$^2$, intestinal bacteria were present in small numbers, and the germs from *Pseudomonas* genus were absent.

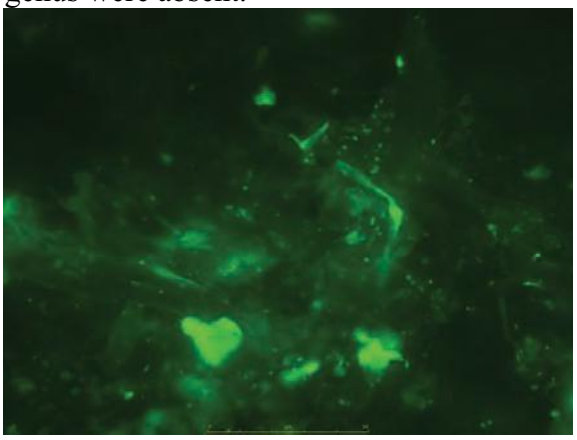

**Fig. 1 . Microbial biofilm from the coccigeal region**

In surface samples from cattle carcasses from retail unit (VA 1) the biofilms presence in thoracicoabdominal region, the chest and costal region was founded. The overall level of contamination of carcasses was $10^3$-$10^4$ cfu/cm$^2$, the number of enterobacteria was 23.370 cfu/cm$^2$, and the numbers of germs from genus *Pseudomonas* have exceeded the 20 cfu/cm$^2$.

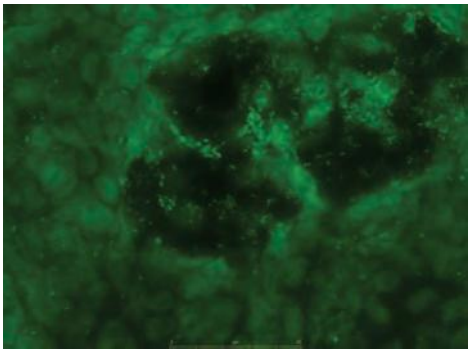

**Fig. 2. Microbial biofilm from the bovine carcasses surface (toracoabdominal region )Col. acridine orange, fluorescent examination 63x**

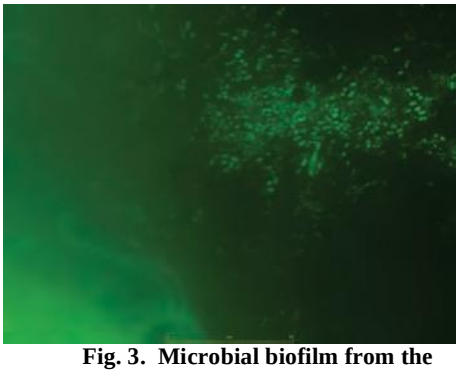

**Fig. 3. Microbial biofilm from the bovine carcasses surface (coast region )Col. acridine orange, fluorescent examination 63x**

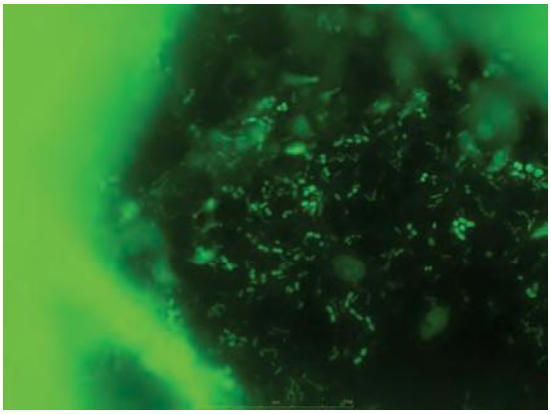

**Fig. 26. Microbial biofilm from the bovine carcasses surface (breast region )Col. acridine orange, fluorescent examination 63x**

The ATP level by Lumitester method recorded values was exceeded, in all samples where the biofilm was isolated, the value of 200 URL/cm$^2$. The results sustain the existence of a positive correlation between the presence of biofilm, microbial load and the amount of ATP.

## 3. CONCLUSIONS

3.1. On the surface of pork carcasses and cut pieces (at least three days aging) microbial biofilm has been emphasized in a relatively high number of samples (29.4%).

3.2. On the surface of cattle carcasses the microbial biofilm presence was put into evidence in thoracicoabdominal region, the chest and costal region (42% of samples).

3.3. On the surface of poultry carcasses has not revealed the presence of microbial biofilms in any of the carcass surface regions.

3.4. The overall level of contamination in areas where it emphasized the presence of microbial biofilms, has fluctuated on average from $10^2$-$10^4$ cfu/cm$^2$.

3.5. Method using the device Lumitester could represent a rapid method for monitoring of the carcasses microflora

## ACKNOWLEDGEMENTS

This study was supported by the project no. 1101 from 2009**,** COD 902 financed by Ministry of Education, Research and Innovation – National University Research Council We also thank the staff of the processing plant and retail unit involved in this study for facilitating access.

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# EXTENSIVE RAISING SYSTEM INFLUENCES THE NON-SPECIFIC IMMUNE REACTIVITY IN BOVINE

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**Keywords:** phagocytosis, immune globulins, immune complexes, extensive farming, bovine

## SUMMARY

Monitoring the changes of the immune system in various environments could provide useful information for dairy cow breeders to ensure optimal rearing and welfare conditions for their animals. The research envisaged the global influence of household farming of 12, Romanian Spotted dairy cows aged 5 to 10 years, on humoral and cell-mediated immunity at innate level. Total immune globulin (tIg) and circulating immune complexes' (CIC) levels were quantified by precipitation techniques (2.4%o zinc sulphate and 4.5% polyethylene glycol, respectively), red spectrophotometrically and expressed as conventional units. Phagocytosis was monitored by an *in vitro* carbon particle inclusion test over time, with readings of optical densities by spectrophotometry ($\lambda$=535nm, d=0.5cm). Untreated blood and alcohol as well as alcoholic *Silybum marianum* extract treated variants were incubated with India ink at 37°C for 0 (t0), 25 (t1) and 50 (t2) min. Optical density readings were converted to a log2 scale and phagocytic index was calculated as the negative of the slope of the regression of optical density (log2)(t0-t1, t1-t2). tIg and CIC values were relatively high, when compared to physiological values (40.1±16.6 and 7.50±5.16, respectively) but they stayed lower than those encountered in intensive raising. Phagocytosis was diminished both in control and treated variants, compared to normal values. The *Silybum marianum* extract exerted a negative effect on innate cell mediated activity. Extensive farming apparently had a more pronounced negative effect on innate cell-mediated than on innate humoral immunity in dairy cows.

Dairy cows are raised in Romania in both intensive and extensive systems, but apparently the intensive system actes on the individual in a way that often leads to economic failure (Georgescu, 1995). As opposed to this, in special organic conditions, on small farms, the food products obtained – meat and milk – have the highest nutritional and technological quality but the final product's price is very high. Thus, the household rearing system of dairy cows that could seam dreamlike for the animals, could pose serious economic problems to the owners (Morar *et al.*, 2005; Man *et al.*, 2002; Onaciu, 2006).

Regardless of the raising system, the individual confronts microbial aggression; therefore the integrity of the immune system, one of the most sensitive radars of the body might be crucial. Monitoring the changes at this level may provide useful information for the dairy cow breeders to ensure optimal rearing and welfare circumstances for their

animals. The aim of this work was the monitoring of the influence of household raising of dairy cows on the innate immunity as a measure of disease resistance.

## 1. MATERIAL AND METHODS

**Animals and protocoles**. The experiment was carried out on a group of dairy cattle from Sibiu County, raised in extensive (household) system, which represents the traditional way of raising dairy in Romania. The barns used are of reduced capacity, the same building may shelter other animal species too (horses, pigs, buffalos, poultry and so on) of different ages. The barns were built of bricks, showing various sizes, according to the sheltered animal numbers and species. The barns are roofed with tiles or eternit (fiber cement). Animals were kept and hay was stored under the same roof, in separate divisions of the building. The height of barns was usually lower than required by standard regulations. The buildings had one or two small windows, usually dirty, leading to insufficiency natural illumination. The artificial lighting had low intensity, being provided by only one or two electric light bulbs placed on the barn ceiling.

Feeding was done by manpower, with green forage in the summer and hay in the winter. Sometimes grain supplements (cornmeal and bran) were given as well as succulent feed (beetroot), the quantity and the administration frequency depending on the economic possibilities of the owners. Cows were watered individually, from buckets or in some households animals were moved outside to drink from a trough. The usual bedding material was straw. Manure was cleaned usually once, in the morning, by the owner. Cows were milked manually, in buckets, twice a day, in the morning and in the evening.

In the majority of the barns the microclimatic and hygiene were precarious; the disinfectants used for barns' sanitation were not adequate. The bleaching of the buildings was performed once a year, after the beginning of the pasture season or maximum twice a year, in the spring and in the fall.

The working protocol included a single blood sampling in 12 cows aged between one and 10 years. Blood samples were taken in the early morning hours, collecting 4,5 mL of blood from each of the cows, in sterile test tubes containing heparin (50 UI/mL) in order to perform carbon particle inclusion test and on a pro-clotting gel for serum.

**Total immune globuline (Ig) measurements.** Part of the collected blood was allowed to clot for 30·min at 37°C and then centrifuged at

1308·*g* for 10·min. Sera were removed and kept at –20°C until tested. A volume of 3.3·µl of serum was mixed with 196.7·µl of a 0.024% barbital buffer zinc sulphate solution and allowed to precipitate for 30·min at room temperature (22–23°C). Optical density (ODU) was read spectrophotometrically (λ=475·nm, d=0.5·cm) and multiplied by 100 to convert the values in Vernes degrees.

**Circulating immune complexes (CIC) measurements.** A 4.2% polyethylene glycol (PEG) solution in borate buffer was used as the precipitating agent, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. Volumes of 193.4·µl of borate buffer and PEG solution, respectively, were mixed with 6.6· µl samples of the serum, for each sample. The samples were allowed to precipitate at room temperature (22–23°C) for 60·min, then read spectrophotometrically (λ=450·nm, d=0.5·cm)(multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). CIC concentrations, (ODU) were calculated by subtracting the value of the control (serum + buffer) from that of the PEG precipitate and multiplying the difference by 1000.

**Carbon particle inclusion test**. Phagocytic cells engulf inert particles such as carbon dueto the defensive capacity of these cells. 0.50·ml portions of heparinized blood were mixed with 2·µl of supernatant of India ink, which were obtained by centrifugation at 1308·***g*** for 40·min (CN-2060 microprocessor control centrifuge, Hsiangtai Machinery Industry Co. Ltd, Taiwan). 0.15·ml of the mixture were transferred immediately to 2·ml of saline and the rest was incubated for 25·min at 37°C. Another 0.15·ml sample was transferred to saline and the incubation was continued to 50·min, repeating the operation. All tubes containing saline, blood and ink were centrifuged at 419·***g*** and the supernatants were read spectrophotometrically (l=535·nm, *d*=0.5·cm). There was a decrease in absorbance with time as carbon was phagocytized. Phagocytic activity index was calculated as the difference between the natural logarithms (log2) of the optical densities of the phagocytosis at 0–25·min and 25–50 min..

In parallel, following the same working protocol, variants also were carried out, processing blood samples treated previously with *Silybum marianum* extract and using 70° ethanol, as control substance for the herbal extract (Ghergariu *et al.*, 2000).

## 2. RESULTS AND DISCUSSION

Microbial aggression induced an inflammatory response followed by adaptive responses of both humoral and cell-mediated brances of the immune system. The pooled level of immune globulins represent an indicator of the overall, multifunctional humoral reactivity (Roitt and Delves, 2001; Zahao, 2006). Opsonins bind with microbes and enhance their clearance. Circulating immune complexes (CIC) are aggregates of large dimensions which can be precipitated with different chemical compounds, using particulary polimers with high molecular weight as it is the polyethylene glycol (PEG) (Ghergariu *et al.*, 2000).

Total immune globulin concentrations (Table 1) in dairy cows kept in extensive rearing system were close to physiological ones, with no statistically significant differences. This indicates a mild influence of the raising environment on this immunological parameter. Nevertheless, due to poor hygiene, one would expect a higher level of total Ig; this could be balanced by the negative inflyuence of stresfull factors (social stress, nutritional stress, environmental stresses and more).

***Table 1***

**Mean values of total serum immunoglobulins in dairy cows kept in extensive raising system**

| Sample number | ODU | Vernes degrees |
|---|---|---|
| Mean | 0, 401 | 40,1 |
| Standard deviation | 0,166 | 16,6 |

ODU = Optical Density Units

Starting from the immunologic concept which states that microbial aggression induces an immune response translated as the synthesis of gamma globulins with antibody activity, the dosage of total immunoglobulin levels was developed as a diagnostic technique (Roitt *et al.*, 1996). The immunoglobulin levels are certainly conditioned not only by the synthesis but also by consumption, practically by the rate of immune complex formation and clearance (Turner,1994; Goldsbi *et al.*, 2001).

The synthesis of circulating immune complexes represents a way to eliminate biological aggressors from the body. Moreover, immune complexes have an important role in the activation of the complement, augmenting the clearance capacity of the macro-organisms (Bajaj *et al.*, 1990). Immune complexes are formed by recognition and coupling of antibodies with their inductor antigens (Goldsbi *et al.*, 2001). The

development of these aggregates is controlled by the antibody titers in the biologic liquids, the avidity of these specific effectors and the clearance rhythm of the formed complexes (Roitt and Delves, 2001). The immune complexes are cleared, without any further consequences. If their formation rate is higher, their depositing in cell membranes could enhance the activation of the complement, subsequently other mediators, which could cause severe cell damage and sometimes type III auto-immune diseases. It is explicable therefore, in the context of an immunologic survey, how the determination of the immune complexes' concentration becomes important.

Levels of immune complexes quantified during the experiment were indicated in Table 2. The mean values recorded for the circulating immune complexes do not exceed the physiological limits (Ghergariu *et al.*, 2000).

***Table 2***

**Mean values of the serum circulating immune complexes in dairy cows kept in extensive husbandry system**

| Number | PEG (ODU) | Buffer (ODU) | CIC (Units) |
|---|---|---|---|
| Mean | 0,05 | 0,04 | 7,50 |
| Standard deviation | 0,02 | 0,02 | 5,16 |

ODU = Optical Density Units

Phagocytosis represents one of the important aspects of the anti-infective protection. Functional tests can identify, in the case of a secondary neutrophilic deficit, the disturbances that occur in different stages of phagocytosis: chemo taxis, interaction with opsonized particles, ingestion, development of the respiratory shunt, destruction and digestion of micro-organisms (Peretianu *et al.*, 1998).

The results of the phagocytic activity recorded in the group of dairy cows kept in extensive (household) breeding are shown in table 3.

The values recorded for the control samples and *Silybum marianum* treated variant were relatively close, while the alcohol treated variant recorded the highest indicators. Spontaneous phagocytosis was positive in the second reading period and it was strongly inhibited by the vegetal extract used.

***Table 3***

**The phagocytic activity values during the study period in dairy cows bred in extensive system (nl)**

| | Control sample | | Variant treated with alcohol | | Variant treated with *Silybum marianum* | |
|---|---|---|---|---|---|---|
| | nl0'-nl25' | nl25'-nl50' | nl0'-nl25' | nl25'-nl50' | nl0'-nl25' | nl25'-nl50' |
| Mean | -0.078 | 0.011 | -0.049 | -0.001 | -0.090 | -0.051 |
| SD | 0.21 | -0.29 | 0.01 | 0.01 | 0.03 | 0.01 |

nl = Natural logarithm; SD = Standard deviation

## 3. CONCLUSIONS

3.1. The traditional, extensive (household) rearing of dairy cows indicated total Ig, circulating immune complexes and phagocytosis levels close to physiological limits, as a consequence of that relatively enclosed housing system, with a mildly changing microflora.

3.2. There was no positive effect of the alcoholic *Silybum marianum* extract on phagocytic, the active principles acting rather inhibiting than stimulating.


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# CHANGES OF ADAPTIVE CELL-MEDIATED IMMUNTY FOLLOWING THE *IN VITRO* VEGETAL EXTRACT TREATMENT IN EXTENSIVELY RAISED DAIRY COWS

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**Keywords:** plant extracts, dairy cows, adaptive immunity, extensive farming

## SUMMARY

Lymphocyte activation or their stimulation refers to a correlation of their *in vitro* activity with phenomena regularly happening *in vivo*, as the host interacts with an antigen. Thus, the *in vitro* blast transformation capacity serves as a measure of the reactive potential of the cells and also as a tool to monitor the efficacy of various components (drugs, antigens, other active compounds) in modulating this response. The investigations were carried out on 12 Romanian Spotted dairy cows aged 5 to 10 years, kept on private households, considered extensive farming conditions, to evaluate the efficacy of alcoholic extracts of milk thistle (*Silybum marianum*), common sea-buckthorn (*Hippophae rhamnoides*), bilberry (*Vaccinium myrtillus*), thyme (*Thymus vulgaris*) and medicinal aloe (*Aloe vera*) on adaptive cell-mediated immunity. Blood samples were collected on heparine (50UI/ml), transferred in culture medium (RPMI 1640, 1;4) and distributed in duplicate as 200 microliter aliquots, in 96 well plates. After 60 h of incubation at 37 °C, glucose levels in the culture supernatants were quantified by an orto-toluidine colorimetric test. Blast transformation indices were calculated as percentages of glucose consumed from the initial amount present in the culture medium. Statistical parameters were calculated by use of Excel program. There were no significant differences between the experimental variants, except for those treated with *Vaccinium myrtillus* and *Thymus vulgaris* ($p<0,05$) versus PHA and alcohol treated variants (36,68± 19,98% and 35,36±10,67% versus 58,26±25,41% and 58,96±15,25% respectively). Although the stimulation index of the control culture was a physiological one (56,78±12,16%) none of the extracts had a stimulating effect. Furthermore, some of the extracts (*Vaccinium myrtillus* and *Thymus vulgaris*) exerted a strong inhibiting effect, showing that some of the plants found on pastures could negatively influence the adaptive cell mediated protection in extensively raised dairy cows.

The knowledge and understanding of optimal breeding and productive conditions are of equal concern for veterinarians and dairy cow breeders (Onaciu G., 2006).

Researches on the effects of several herbal extracts on the immunity in farmed animals concern the effects of active principles on both innate and adaptive effectors of the immune system. Not only these, but also whole extracts could provide an efficient therapeutic alternative, due to their higher biological availability (Liu and Ng, 2000, Devasagayam and Sainis, 2002, Dewick, 1997).

The research aimed to monitor the effect of certain vegetal extract on the adaptive cells of the immune system and render those according to their efficacy.

## 1. MATERIAL AND METHODS

The experiment was carried out in a group of dairy cattle reared extensively, in a system which represents the traditional way of dairy cattle raising in Romania. The cows' ages were between one and 10 years.

**Animals and protocoles**. Twelve, Romanian Spotted milking cows, kept in households in the region of Sibiu, were subjected to this study. The animals were accommodated in traditional brick or wooden barns, sometimes together with other farmed species (swine, horses, poultry, etc). natural light was scarce, the hygienic conditions being frequently disobeyed. The animals were fed and watered individually, manure was collected once a day, in the morning. The usual bedding material was straw. Cows were milked manually, in buckets, twice a day, in the morning and in the evening.

The working protocol included a single blood sampling in 12 cows aged between one and 10 years. Blood samples were taken in the early morning hours, collecting 4,5 mL of blood from each of the cows, in sterile test tubes containing heparin (50 UI/mL) in order to perform the *in vitro* balst transformation test.

*Blast transformation test (Ghergariu et al., 2000).* The blast transformation capacity of leukocytes was tested on whole blood cultures by use of RPMI 1640 (Sigma-Aldrich) culture medium, with 5% fetal calf serum (FCS)(Sigma-Aldrich). The blood samples were diluted 1:4 with the medium, distributed in 96 well plates in 0.2 ml aliquots. PHA M (Sigma-Aldrich.)(1µl/well) was used as a standard mitogen. 70°alcohol and various alcoholic plant extracts: milk thistle (*Silybum marianum*), common sea-buckthorn (*Hippophae rhamnoides*), bilberry (*Vaccinium myrtillus*), common thyme (*Thymus vulgaris*) and medicinal aloe (*Aloe vera*) were used *in vitro* to monitor their influence on blast transformation. All variants were performed in duplicate, using 1.5 µl of compound / well. The plates were incubated at 37°C in a 5% CO2 atmosphere for 60 hours. Cell growth was estimated by spectrophotometrical (SUMAL PE2, Karl Zeiss, Jena) measurement ($\lambda$=610 nm, d=0.5 cm) of the glucose residue in a colorimetric orto-toluidine test (ICCF, Bucharest). Blast transformation indices were

calculated as percentages of the consumption versus the initial glucose concentration of the RPMI 1640 medium.

The data were statistically interpreted, by calculating mean values and standard deviations as well as the statistical significance of the differences by use of Student's t test.

## 2. RESULTS AND DISCUSSION

Lymphocyte activation or their stimulation refers to a correlation of their *in vitro* and *in vivo* activities, whenever there is an interaction of the macro-organism with an antigen (Peretianu and Saragea, 1998; Vanmiert, 1991). The transformation of lymphocytes is the term used in scientific literature to describe the morphological changes which take place at the small, "latent" lymphocytes' levels, when these are transformed in blast cells (Baiteriakova *et al.*, 1982). Blastogenesis refers to the presence of large, pyroninophilic cells in cultures treated either with mitogens or antigens (Roitt *et al.*, 2001, Roitt and delves, 2001; Tizard, 1996; Dumitru et al., 1996).

By use of the blast transformation test the proliferative response of lymphocytes towards classical mitogens (PHA and Con A) as well as to several alcoholic herbal extracts could be assessed.

Comparing the obtained results it could be observed that, in cell cultures from extensively raised dairy cows, the *in vitro* used alcohol had a slightly stimulating effect, while the alcoholic plant extracts acted inhibiting.

***Table 3***

**Mean values of the stimulation indices obtained in the blastic transformation test in the extensively bred dairy cows (%)**

| | C | PHA | Alc | Sm | Hr | Vm | Tv | Av |
|---|---|---|---|---|---|---|---|---|
| Mean | 56,78 | 58,26 | 58,96 | 55,22 | 52,26 | 36,68 | 35,36 | 52,73 |
| SD | 82,16 | 85,41 | 85,25 | 88,90 | 88,84 | 89,98 | 90,67 | 85,37 |

**C = control sample, untreated; PHA= variant treated with phytohemagglutinin; Alc = variant treated with 70° alcohol; Sm = variant treated with *Silybum marianum*; Hr = variant treated with *Hippophae rhamnoides*; Vm = variant treated with *Vaccinium myrtillus*; Tv = variant treated with Thymus *vulgaris;* Av = variant treated with *Aloe vera*; SD = standard deviation**

Among the used alcoholic extracts, the bilberry (*Vaccinium myrtillus*) and the thyme (*Thymus vulgaris*) extracts induced the lowest stimulation indices while the *Silybum marianum* extract recorded the

highest stimulation index yet inferior to the indices obtained in the control sample or in the PHA treated varinat. The sea-buckthorn (*Hippophae rhamnoides*) and the medicinal aloe (*Aloe vera*) extracts showed average values of the stimulation indices which were comparable to each other, lower than those obtained for the control but higher than those recorded for the variants treated with bilberry (*Vaccinium myrtillus*) and common thyme (*Thymus vulgaris*) extracts.

There were no significant differences between the experimental variants, except for those treated with *Vaccinium myrtillus* and *Thymus vulgaris* ($p<0,05$) versus PHA and alcohol treated variants (36,68± 19,98% and 35,36±10,67% versus 58,26±25,41% and 58,96±15,25% respectively). Although the stimulation index of the control culture was a physiological one (56,78±12,16%) none of the extracts had a stimulating effect. Furthermore, some of the extracts (*Vaccinium myrtillus* and *Thymus vulgaris*) exerted a strong inhibiting effect.

## 3. CONCLUSIONS

3.1. The results of the blastic transformation test show that the alcoholic plant extracts used *in vitro* had inhibitory effect comparative with the control and alcohol treated variants.

3.2. There were no significant differences between the experimental variants, except for those treated with *Vaccinium myrtillus* and *Thymus vulgaris* ($p<0,05$) versus PHA and alcohol treated variants. Although the stimulation index of the control culture was a physiological one none of the extracts had a stimulating effect.

3.3. Some of the extracts (*Vaccinium myrtillus* and *Thymus vulgaris*) exerted a strong inhibiting effect showing that some of the plants found on pastures could negatively influence the adaptive cell mediated protection in extensively raised dairy cows.

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# CORELATIONS BETWEEN PLASMATIC CORTISOL LEVEL EVOLUTION AND THE MAIN HEMATOLOGICAL PARAMETERS IN THE EWE DURING THE ESTROUS CYCLE

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**Key words:** plasma cortisol, hematological parameters, estrous cycle, ewe.

**SUMMARY**

The work studied the relationship between the evolution of the plasma cortisol levels and some blood parameters in ewes during the estrous period of the sexual cycle compared to barren ewes. The monitoring period ranged between the first day of heat apparition and the day of the heat disappearance, over of four days until the heat period finished. A peak of the cortisol level was found in the first day of the estrous period (19.88 ng/mL of plasma), then the cortisol level decreased to values compared to the control ewes (4.18 ng/mL of plasma). Blood changes which followed the evolution of cortisol levels were dominated by eritrocytosis, increase of hemoglobin level, increase of hematocrit, and leukocytosis with lymphopenia.

During the estrous cycle in ewe, as in other mammals, a series of hormonal events take places. These hormonal events control the phases of the estrous cycle. In the same time, other hormonal events control the afferent metabolic processes that occur along the sexual cycle. One of the hormones with major involvements in the sexual cycle is the adrenal cortisol. Cortisol is one of the hormones responsible to the correlations between metabolic modifications with the energetic requirements which characterize each phase of the estral cycle. The metabolic events reflects them self in the evolution of the hematological parameters. In this work we proposed the monitoring of the evolution of the plasmatic levels of cortisol and the main hematological parameters in a number of ewes in the period of the estrous of the estral cycle.

## 1. MATERIAL AND METHODS

For issuing of these objectives it has been established experimentally homogenous group of two years adult ewes, clinically healthy, which was diagnosed physiological state of heat. Experimental group was represented by Karakul, Spanca and Tzigae breed ewes. Diagnosis of heat conditions was done on clinical evidence. In parallel control group was consisted of barren ewes, which are practically in diestrum phase (or anestrous) of the estrous cycle. Serum cortisol was determined using an

enzyme immunoassay method. Hematological parameters were determined based on usual clinical laboratory methods.

Blood cortisol level was monitored beginning immediately with the development of heat, during deployment (manifestation) of heat and two days after their disappearance in order to reveal differences from pre-estrous period.

The first sampling was done on September 12$^{th}$ 2009, when it was noticed the onset of mating season, that heat, of the flock memberships. Biological sampling were continued until signs of heat loss of each ewe, doing two blood samples daily, one in the morning around 9 a.m. and another one in the evening around 9 p.m. During the experimental period, the animals were monitored in terms of the evolution of weight and health.

The data were statistically processed and presented as average ($\bar{X}$) ± standard error of mean: ($s_{\bar{x}}$): $\bar{X} \pm s_{\bar{x}}$. Significance of difference between groups was calculated using the Student *t* test. Differences between groups were categorized according to the P value (null hypothesis probability) as no-significant (when $P>0.05$), significant (when $P<0.05$), distinct significant (when $P<0.01$) or very significant (when $P<0.001$), according to Boyd [1].

## 2. RESULTS AND DISCUSSIONS

From the data analysis presented in Tables 1 and 2 it results that in the case of the control group, represented by adult ewes aged two years in a state of anestrous, plasma cortisol levels remained relatively constant, fluctuating between 3.65 ng/mL of plasma at the start of monitoring period and 4.92 nm/mL of plasma at the end of this period. There is probably only a slight increase associated intensification of distress in the flock, which was generated by the installation (an increasing agitation) in other ewes. At the same batch there are generally reduced values in the morning (Morn.) and higher in the evening (Evn.), which can be attributed to a normal diurnal rhythm of secretion of this hormone.

*Table 1*

**Results of the evolution of the plasmatic concentration of cortisol (in ng/mL of plasma) in eight control ewes which were in anestrous period of the estrous cycle, aged two years, for five consecutive days, between September 12th and September 16th 2009**

| No | Identity number | Sampling of the blood/values in ng/mL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | First day | | Second day | | Third day | | Fourth | | Fifth day | |
| | | I (Evn.) | II (Morn.) | III (Evn.) | IV (Morn.) | V (Evn.) | VI (Morn.) | VII (Evn.) | VIII (Morn.) | IX (Evn.) | X (Morn.) |
| 1 | No mark | 3.34 | 4.54 | 2.76 | 3.90 | 4.54 | 4.79 | 3.87 | 3.90 | 5.34 | 5.95 |
| 2 | RO1044811755 | 4.09 | 5.00 | 4.43 | 4.54 | 3.54 | 4.43 | 5.56 | - | 3.98 | 4.50 |
| 3 | RO1044811754 | 4.00 | 4.21 | 4.87 | 6.46 | 5.09 | - | 2.54 | 4.32 | 5.65 | 4.32 |
| 4 | RO1044811208 | 2.98 | 4.21 | 4.06 | 4.66 | 3.45 | 4.05 | 4.21 | 4.88 | 3.38 | 5.11 |
| 5 | No mark | 3.33 | 3.67 | 3.53 | 4.00 | 3.32 | 4.11 | 3.65 | 3.95 | 3.54 | 3.65 |
| 6 | RO1044811511 | 4.21 | 5.23 | 2.43 | 3.21 | 5.43 | 4.20 | 4.43 | 4.90 | 5.21 | 5.41 |
| 7 | RO1044811701 | - | 4.44 | 4.05 | 5.55 | 4.55 | 4.88 | 4.54 | - | 5.21 | 5.54 |
| 8 | $\bar{X} \pm S_{\bar{x}}$ | 3.65± 1.54 | 4.47± 1.09 | 3.73± 1.65 | 4.61± 2.02 | 4.27± 2.11 | 4.41± 1.65 | 4.11± 2.55 | 4.39± 1.65 | 4.61± 1.06 | 4.92± 1.54 |
| 9 | Max. val. | 2.98 | 3.67 | 2.43 | 3.21 | 3.32 | 4.05 | 2.54 | 3.9 | 3.38 | 3.65 |
| 10 | Min. val. | 4.21 | 5.23 | 4.87 | 6.46 | 5.43 | 4.88 | 5.56 | 4.9 | 5.65 | 5.95 |

***Legend:***
**Evn. = Evening**
**Morn.= Morning**
**Max. val. = maximal values**
**Min. val. = minimal values**

***Table 2***

**The evolution of the plasmatic concentration of cortisol (in ng/mL of plasma) in eight ewes which were in estrous period, aged two years, for five consecutive days, between 12th September and 26th September 2009**

| No. | Identity number | Sampling of the blood/values in ng/mL | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | First day of sampling (the MOMENT of estrous appearance) | Second day of blood sampling | | Third day of blood sampling | | Fourth day of blood sampling | | Fifth day of blood sampling | |
| | | I | II (Morn.) | III (Evn.) | IV (Morn.) | V (Evn.) | VI (Morn.) | VII (Evn.) | VIII (Morn.) | IX (Evn.) |
| 1 | RO1044811790 | 21.65 | 22.43 | 21.32 | 12.20 | 12.89 | 4.67 | 5.32 | 3.33 | 4.05 |
| 2 | RO1044811211 | 12.44 | 12.90 | 14.87 | 8.21 | 9.43 | 5.55 | 5.98 | 3.87 | 3.90 |
| 3 | RO1044811587 | 14.56 | 15.29 | 15.06 | 10.05 | 11.98 | 6.32 | 5.99 | 5.45 | 5.95 |
| 4 | RO1044811723 | 26.71 | 24.20 | 23.11 | 16.50 | 15.43 | 5.65 | 7.54 | 4.65 | 5.76 |
| 5 | RO1132345210 | 16.30 | 14.86 | 15.76 | 11.43 | 10.89 | 6.66 | 6.98 | 3.77 | 5.61 |
| 6 | RO1044811700 | 15.33 | 17.73 | 17.74 | 9.05 | 10.05 | 5.04 | 5.88 | 3.65 | 4.04 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | RO1044811767 | 18.52 | 31.81 | 29.50 | 11.33 | 11.43 | 4.59 | 5.90 | 4.54 | 6.32 |
| 8 | $\bar{X} \pm s_{\bar{x}}$ | 17.93± 6.09 | 19.88± 5.55 | 19.62± 7.43 | 11.25± 3.90 | 11.72± 5.76 | 5.49± 2.87 | 6.22± 3.34 | 4.18± 3.90 | 5.09± 2.54 |
| 9 | Max. val. | 12.44 | 12.9 | 14.87 | 8.21 | 9.43 | 4.59 | 5.32 | 3.33 | 3.9 |
| 10 | Min. val. | 26.71 | 31.81 | 29.5 | 16.5 | 15.43 | 6.66 | 7.54 | 5.45 | 6.32 |

*Legend*: as in Table 1.

Unlike ewes in anestrous, ewes entering the heat, when heat splitting, blood tests relived a high concentration of cortisol, on average amounted to 17.93 ng/mL, which is worth about five times higher than for ewes in sexual rest (anestrous). The next day after splitting heat, the heats were fully expressed in terms of clinical; there was a slight increase in cortisol in the morning and also in the evening. Average values obtained on consignment of ewes were monitored on average amounted to 19.88 ng/mL in the morning and 19.62 ng/mL following splitting the evening heats. The average value of 19.88 was a peak of cortisol secretion in the coming days because it was found a decreased of secretion. On day 3$^{rd}$, plasma cortisol levels decreased to 11.33 to 11.43ng/dL, leveling off at minimum values, comparable to the witness, after the fourth day: 4.18 ng/mL in the evening, respectively 5.09 ng/dL in the morning.

It is well known that the secretion of glucocorticoids has circadian rhythms, with a minimum at midnight and early morning and a maximum in anticipation of awakening. Circadian variations in the secretion of glucocorticoids have been explained based on rhythmic changes in the sensitivity of hypothalamic CRH-secreting cells. Their sensitivity decreases to the inhibitory feedback action of cortisol, in the early morning, leading to increased secretion of CRH, ACTH and cortisol. As the day progresses, sensitivity to cortisol increase, so the secretion of CRH, ACTH and cortisol decreases. The precise mechanism by adjust the sensitivity of CRH-secreting cells is not, however, fully explained [2, 4].

Same high values were found in pregnant ewes by the same authors [5, 7].

Parallel with the evolution of the plasma cortisol level, the ewes were monitored for the main morphological evolution of the blood. The results are presented in Table 3 for the control group of ewes (in anestrous) and in Table 4 for the ewes which came into heat. The data analysis presented in these tables show that in the short period of heat ewes, generally amounting 24-36 hours, and morphological blood frame showed a number of significant changes as it following.

*Table. 3*

**The evolution of the main morpho-hematological parameters in a number of eight barren ewes (considered as control group), two years aged, between September 12th and 16th, 2009. Tha data are expressed as mead ± standard error of mean**

| No. | Item | The chronologic moments of blood sampling vs the moment of the beginning of the estrous | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | The moment of the start of the estrous | 12 hours later | 24 hours later | 36 hours later | 48 hours later | 72 hours later | Four days hours later |
| 1 | Leucocytes - $10^3/mm^3$ | 14.0± 1.3 | 14.4± 2.1 | 15.2± 3.2 | 14.2± 1.5 | 16.3± 2.3 | 15.0± 2.0 | 15.7± 3.2 |
| 2 | Erythrocytes (N) – $10^6/mm$ | 8.4 ± 0.13 | 8.3 ± 1.1 | 7.7 ± 0.4 | 7.9 ± 1.0 | 9.6 ± 0.6 | 7.5 ± 2.2 | 7.1 ± 2,0 |
| 3 | Hemoglobin (Hb)– g/dl | 8.5± 2.1 | 8.9± 0.4 | 9.5± 2.5 | 9.4± 1.5 | 9.0± 3.0 | 9.9± 1.5 | 9.8± 2.9 |
| 4 | Hematocrit (Ht)- % | 40.3± 4.3 | 38.5± 4.3 | 36.0± 3.5 | 37.4± 10.4 | 36.5± 8.5 | 34.5± 5.5 | 37.0± 2.3 |
| 5 | Platelettes – $10^3/mm^3$ | 1.454± 121 | 1.211± 76 | 1.134± 211 | 1.832± 254 | 1.500± 231 | 1.832± 77 | 1.404± 321 |
| 6 | Lymphocytes (%) | 54.4± 21.0 | 46.9± 6.4 | 39.0± 5.4 | 53.1± 5.8 | 43.8± 5.4 | 46.3± 12.5 | 47.9± 6.5 |
| 7 | Monocytes (%) | 6.3± 2.1 | 6.5± 1.4 | 7.4± 0.5 | 7.9±0. 4 | 9.6±2.1 | 6.2±2.2 | 8.4±1.5 |
| 8 | Granulocytes (%) | 30.4±4.4 | 38.1±3. 2 | 43.6±6 .7 | 29.4±1 .9 | 40.8±6. 5 | 40.0±4. 3 | 32.9±9.3 |
| 9 | Eosinophils | 2.7 ±0.4 | 4.5±0.5 | 5.0±0. 4 | 7.6±1. 4 | 12.8±3. 0 | 9.5±2.8 | 12.6±3.6 |

As the number of red blood cells from the anestrous ewes group, it ranged between 5.55 and 6.50x $10^6$/mmc without significant changes during the monitoring period (September, 15$^{th}$ to 21$^{st}$). In what it concerning the consignment of ewe during estrous, there was an increase in the number of red blood cells, expressed at an average of 8.45 x $10^6$/mmc blood when splitting decreasing heat 12x $10^6$/mmc blood after four days of heat splitting. Increasing the number of erythrocytes in heat group of ewes is matched by increased hematocrit. So if in the control group hematocrit values ranged between 30.5% and 33.7%, without significant changes over that period, the consignment of ewes in the heat it took an average of 40.3% when the heat splitting, decreasing to 37.0% in the next four days.

Another dominant hematological change was the leukocyte formula. In this regard it is noted a decrease in the percentage of eosinophil granulocytes from the witness as it follows. At the control group, the

percentage of eosinophils blood count ranged from 12.0% and 16.6%. In the group of ewes in heat, this percentage was 2.7% on the day of heats splitting, 4.5% in the next day of heat, and the average values of 5.0% and 7.6% in the following days, revealing low values being constant. In the next three days it was found an increase of eosinophils percentage: 12.8%, 9.5% and 12.6%, which indicates a return to normal values, which is characteristic of this species. Evolution of eosinophils percentage follows generally the evolution of plasma cortisol level, revealing a correlation between these investigated parameters.

Also in the leukocyte formula it was a change in the ratio of lymphocytes and granulocytes, respectively, increasing this ratio at the ewes in heat compared to barren ewes. In other words, there was an increase in the number of lymphocytes and a decrease in neutrophils.

*Table 4*

**The evolution of the main morpho-hematological parameters in a number of eight ewes in the estrous period of the estral cycle (experimental group), two years aged, from September 12th to September 16th, 2009. The data are expressed as mean ± standard error of mean.**

| No. | Item | Calendar data of blood sampling/values | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 15 Sept. | 16 Sept. | 17 Sept. | 18 Sept. | 19 Sept. | 20 Sept. | 21 Sept. |
| 1 | Leucocytes - $10^3/mm^3$ | 17.5± 2.1 | 18.2± 3.2 | 18.6± 6.4 | 19.5± 4.5 | 17.3± 2.0 | 16.6± 5.5 | 17.0± 4.0 |
| 2 | Erythrocytes (N) – $10^6/mm$ | 6.06± 0.22 | 5.96± 1.40 | 5.55± 0.45 | 6.54± 1.43 | 6.50± 0.95 | 6.20± 0.78 | 6.12± 1.12 |
| 3 | Hemoglobin (Hb)– g/dl | 7.7± 0.6 | 7.7± 0.5 | 7.9± 1.0 | 8.5± 2.1 | 8.0± 2.4 | 8.2± 0.5 | 7.8± 1.1 |
| 4 | Hematocrit (Ht)- % | 33.7± 9.5 | 30.5± 9.6 | 33.0± 4.7 | 31.5± 10.0 | 33.3± 7.4 | 32.9± 6.5 | 33.0± 12.0 |
| 5 | Plateletes – $10^3/mm^3$ | 1.716± 320 | 1.543± 65 | 1.657± 289 | 1.230± 320 | 1.421± 354 | 1.454± 56 | 1.211± 365 |
| 9 | Lymphocytes (%) | 70.3± 5.0 | 66.7± 6.5 | 65.6± 13.5 | 72.4± 9.0 | 76.5± 9.3 | 71.9± 6.3 | 69.8± 15.4 |
| 10 | Monocytes (%) | 10.1± 2.2 | 8.1± 2.0 | 9.1± 0.5 | 8.9± 0.6 | 9.3± 1.3 | 10.1± 2.1 | 10.6± 3.5 |
| 11 | Granulocytes (%) | 14.9± 3.3 | 25.2± 3.2 | 25.2± 3.1 | 19.8± 5.3 | 15.0± 2.5 | 17.9± 5.0 | 19.5± 3.8 |
| | Eosinophils | 12.6± 3.0 | 15.4± 2.0 | 15.3± 2.2 | 12.5± 1.8 | 16.6± 6.5 | 17.4± 4.5 | 12.0± 2.9 |

## 3. CONCLUSIONS

3.1. Monitoring of plasma cortisol levels in ewe during the estrous period conducted to identifying a level about four times bigger of this blood hormone compared with anestrous ewes.

3.2. Blood changes which followed the evolution of cortisol levels were dominated by eritrocytosis, increase of hemoglobin level, increase of hematocrit, and leukocytosis with lymphopenia.

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# HISTOLOGICAL AND ULTRASTRUCTURAL ASPECTS OF THE LIVER IN EXPERIMENTAL OCHRATOXICOSIS OF BROILER CHICKENS

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**Key words:** ochratoxicosis, chickens, liver, histology, electronomicroscopy

**SUMMARY**

Investigation were conducted on 2 groups, each of 15 broiler chickens. Experimental group received ochratoxine A (OTA) orally, in sunflower oil suspension, daily, for 21 days in doses of 50 µg/kg b.w. Control group (of 15 chickens) received only sunflower oil. 5 chickens from each group were killed after 7,14 and 21 days of the experiment.
Histological exam of liver evidentiated congestion, steatosis and yones of necrosis of hepatocytes and endothelial cells.
Ultrastructural changes of hepatocytes were more evident at 21st day of the experiment. Into the hepatocytes,lipidc vacuolae, fragmentation and dilatation of smooth endoplasmic reticulum, decrease number of ribosomes attached to endoplasmic reticulum, increased number of free ribosomes into the cytoplasm, vacuolar lipidic inclusions, balonised mitochondria with smaller cristae and lipidic droplets into it, and loose of membrane integrity were observed. Glicogenic granulae into the hepatocytes were also observed. The nuclei of the hepatocytes had irregular shape,contained large lipidic vacuolae and electronodense inclusions and into the cytoplasm. Mitochondria were totally dezintegrated and many myeline-like figures were observed. In control group the bile canaliculi showed numerous expansions of the hepatocytes. In experimental groups both the number and the high of microvili were reduced.

Ochratoxicosis is a micotoxicosis determined by a number of metabolites, mainly of Aspergillus ochraceus and Penicillium viridicatum. Ochratoxins produce immunosuppression, a significant fat hepatosis, inhibit glycogenolysis, glycogen being stored in the cytoplasm of hepatocytes (11). Also determine the relative increase of the liver (24.9%), kidney, gizzard (12%), reduced serum total proteins (albumin and globulin) (14).

## 1. MATERIALS AND METHODS

For the experiment, 30 Ross 307 broilers were used, which after one week necessary to adjust to provided living conditions, were separated into two groups of chicken with an average weight of 79,03 ± 0.73 g. The chicks were reared on permanent litter, were assured of age specific microclimate conditions, from 32 ºC, dropping gradually to 24 °C. The first group received daily, by gavage, ochratoxin A (OTA – Sigma Chemical Co.) eluted in sterilized sunflower oil at a dose of 4

µg/chicken, corresponding to 50.62 µg/kg BW and the control group received LM sunflower oil eluant.

Histological examination was made of liver fragments fixed in 10% formalin solution, impregnated in paraffin, sectioned at 5µm and stained by HEA , PAS method and examined under a Olympus CX41 microscope.

Electronomicroscopic investigations were performed using Philips TES transmission electronic microscope. The method involves the following steps: harvest, prefixation (with 2% glutaraldehyde in PBS, 2 hours at 4 ºC), washing, fixation (with 2% osmium tetroxide), washing, drying, staining with uranyl acetate and phosphotungstic acid solution, infiltration, EPON impregnation, polymerization, trimming, ultramicrotom sectioning (sections of 60-150 nm), grid deposition, staining with uranyl acetate and Reynolds solution.

## 2. RESULTS AND DISCUSSION

The hepatocytes of the chicken treated with OTA showed granular degeneration after the first week of exposure and also hypertrophied bile ducts (Fig. 1. a).

After 2 weeks of exposure in the portobiliar spaces appear small clusters of mononucleate cells, bile ducts being reduced. In three of five cases appear intensely PAS positive stained hepatocytes-like cells, which were observed with acinar arrangement. The cord disposition of the hepatocytes disappears (Fig. 1. b). These features are characteristic to hepatoblastoma. Hepatocytes are small, with intense acidophilous cytoplasm, quantitatively reduced and with central nucleus (fig.1. c). We note the presence of pericapillary edema, of stellate, Kupffer and activated endothelial cells, increased in volume.

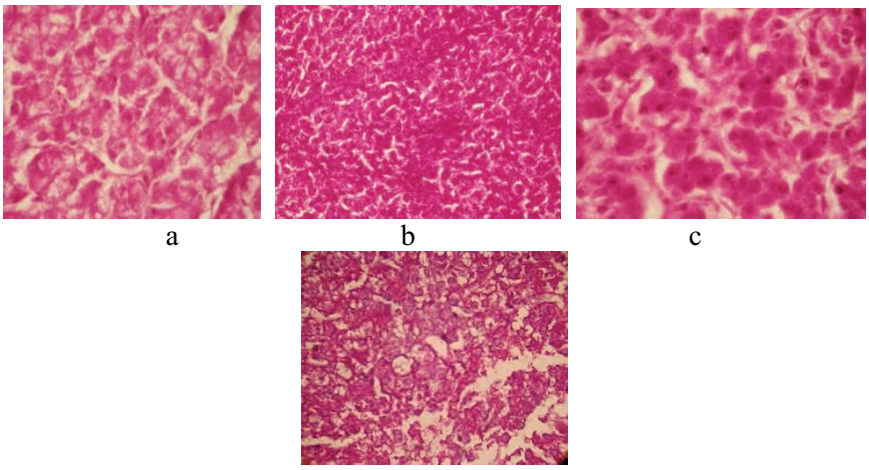


**Fig. 1. Chickens liver after 2 weeks of exposure to OTA (a), 3 weeks - hepatoblastoma characteristic lesions (b, c) areas of necrosis (d).**

After 3 weeks of exposure to OTA in two of the five chicks can be observed areas of necrotic cells in liver (Fig. 1d). Hepatocytes nuclei may be observed in picnosis, cariorexis, cariolisis, and other hypertophiated with 3-5 heterochromatine gatherings.

The mycotoxin is known for its ability to induce oxidative stress (1) and hydrogen peroxide formation. OTA activates cytochrome P-450 enzymes, which converts OTA into 4-hydroxy OTA metabolite with toxic and carcinogenic effects, which would explain the presence of the encountered lesions (2).

The nuclear changes outlined in the groups treated with mycotoxins can be caused by free radical formation as a result of cells toxic injuries. Lipid peroxidation would be the main mechanism of cell destruction through OTA.

Toxic effect of OTA is direct (12), mycotoxins accumulating in a large proportion in the liver (2). In chickens treated 2 weeks with OTA, 400 and 800 ppb/kg, the liver presents a mononuclear cell infiltration in portal area that becomes multifocal after 4 weeks. There were not observed degenerative or vacuolar changes of the liver (4). The synthesis of glycogen in the liver seems to be normal but the tissue mobilization is low due to the inhibition of the responsible enzymes for phosphorylase activation (10).

Perivascular edema observed in the liver may be given by the vascular damage caused by OTA (11, 12, 5). The type of cell death stimulated by the OTA is determined by the dose and exposure time.

Jukk and col. showed that low doses of OTA determine apoptosis and OTA in high doses causes MDCK-C11 cells necrosis. These changes that occurred after one week of i.p. OTA administration, gradually decreased and disappeared within 9 days. Stoev, 2000, describes an unsignificant enlargement of the liver, degeneration and rare hepatic cell vacuolation and also infiltration with mononuclear cells after 42 to 70 days at a dose of 790 μg associated with 2000-5000 mg / kg penicilic acid. Significant morphological changes were reported at doses higher than 2 mg / kg in broilers (7, 9).

The administration for 20 days of a dose of 1 mg / kg body weight produces an increase of the acid phosphatase in the hepatocytes cytoplasm and intercellular space, causing a glycogen cells degeneration (6).

**ULTRAMICROSCOPICAL CHANGES**

Ultrastructural changes of chicken`s hepatocytes in LE are more intense at 14 and 21 days of exposure. Chicken`s hepatocytes after 14 days of exposure to OTA have an increased number of lipid vacuoles, fragmentation, reduction and expansion of SER. The number of ribosomes that are attached to endoplasmic reticulum is small, and of the free ones growing. Mitochondria are bloated with small cristae and inner fat droplets(fig.2a), some loosing membrane integrity (fig.2b). Glycogen granules appear intracytoplasmic. Hepatocytes nucleus show changes consisting in large lipid vacuoles in perinuclear cisterna with inner electrone-dense factions and lipid droplets.

In LE at 21 days of exposure the hepatocytes have total disintegrated mitochondria and numerous myelinated figures in the cytoplasm. Rare normal nuclei alternate with those with chromatolisis. Large intracytoplasmic vacuoles can be observed (Fig. 3a). There were observed stellate, activated cells with an euchromatine rich nucleus, visible nucleolus, lipid droplets in cytoplasm, mitochondria and RER. Endothelial cells shows degenerative changes and capillary basal memebrana has thickened areas (Fig. 3b).

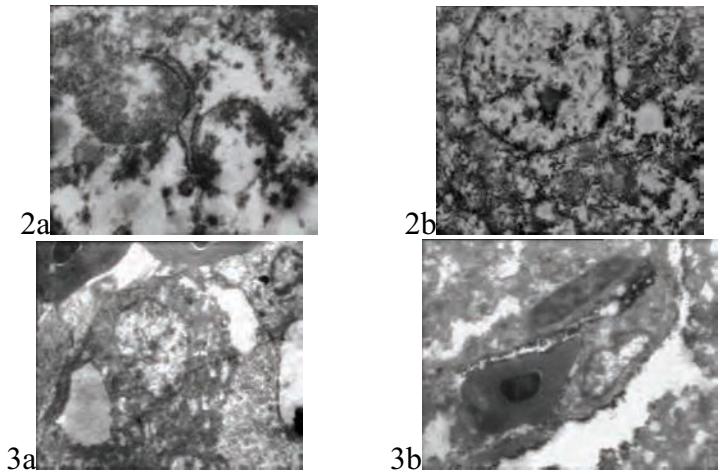


**Fig. 2. Chickens livers after 14 days of exposure to OTA. Bloated mitochondria with reduced cristae (a). Totally disintegrated mitochondria (b).**
**Fig. 3. Chickens livers after 21 days of exposure to OTA. Nucleus with cromatolisis, intracytoplasmic vacuoles (a). Endothelial cells with degenerative changes and capillary basal membrane with thickened areas (b)**

EM examination of the liver shows lipid accumulation which suggests that it is not allowed their entering into the bloodstream and can be interpreted as a disturbance of lipid metabolism.

Focal thickening of the basal membrane and endothelial cells degeneration was another frequent change in OTA intoxicated chickens. Macromolecules synthesis inhibition and oxidative stress are mechanisms specific for this mycotoxin (8); the production of oxygen reactive species by these toxins may have a role in modifying cell division, differentiation, proliferation and metabolism by nonspecific way. Then it induces apoptosis and alteration in other target areas, nucleus, mitochondria, ribosomes, RER (3) and cell membrane that may be responsible for the antagonistic effect.

Lipid accumulation in the liver with lipid depletion in other organs, is the result of rupture of the lipid transport system as a consequence of protein synthesis inhibition at this level. Degeneration of hepatocytes under ochratoxins action is realized by inhibiting protein kinase activity, the enzymatic initiator of glycogen phosphorylation system. Ochratoxin affects primarily the AMPc protein kinase dependent, responsible for initiating the enzymatic cascade that leads to glycogenolysis. Glycolitic enzymes activity is reduced, increased to those of gliconeogenesis, finally observing the degeneration due to accumulation of glycogen (6).

In physiological conditions this cascade is the target of hormones like glucagon and epinephrine which stimulates the adenylate cyclase involved in the production of AMPc secondary messengers (14).

Loss of membrane and cellular organelles integrity may be due to specific inhibition of protein synthesis by OA and to unpaired subsequence of lipoprotein structure of the cell.

On the other hand, OA inhibits oxidative phosphorylation in mitochondria by activating the competitive inhibitors of carrier proteins located on the internal mitochondrial membrane that leads to decreased ATP. The inhibition of protein synthesis and decreased energy production by mitochondria can be considered as the most important factor for degenerative changes of hepatocytes where OA was detected.

OTA-induced ultrastructural changes were studied in bud ducks that received 100 micrograms in 24 hours. These include fat vacuolization, bloated mitochondria with granulations, reducing of ER, isolated, dilated vesicle formation, focal reducing of the ER membrane ribosomes, increasing in the number of ribosomes in the cytoplasm.

Hepatocellular necrosis was described in chickens exposed to OTA accompanied by the endothelial cells bloating (8).

## 3. CONCLUSIONS

3.1. Liver lessions after 2 weeks of exposure to OTA are those of glycogenesis and necrosis after 3 weeks.

3.2.Ultrastructural, hepatocytes after 14 days of exposure to OTA have an increased number of lipid and glycogen vacuoles, fragmentation, reduction and expansion of SER, the degradation of mitochondria, nuclear changes consisting of large lipid vacuoles located in perinuclear cisterna.

3.3.After 21 days of exposure to OTA hepatocytes have totally disintegrated mitochondria, numerous myelinated figures in the cytoplasm and chromatolisis. Endothelial cells present degenerative changes and capillary basal membrane has thickened areas.

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# THE JOINTS OF THE PELVIC LIMB AT RED SQUIRREL

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**Key words**: squirrel, joint, bone, leg.

**SUMMARY**

The stratigraphic studies by dissection of the pelvic limb in the squirrel showed some features of jointing surfaces and ligament structures in strict correlation with the mode of travel required habitat conditions. Thus, the coxofemural joint customizes by a deep coxal jointing cavity and hemispheric aspect of the femoral head. Lengthening of the lateral condyle of the tibia and the lateral meniscus caudally allow, in addition to knee joint flexion and extension, and latero-caudal rotation of the shank. At the tibio-tarso-metatarsal joint, the squirrel has high mobility due the three trochlea of the talus well revealed. The obliquity of the dorsal trochlea produces medio-dorsal displacement of the pelvis in flexion joint of the autopodium.

Lower limb, trough the position under the trunk, plays an important role in the station and propulsion of the body. Following this office, have developed this system and muscles osteoligamentar engines that allow movement (Feider *et al).*

## 1. MATHERIAL AND METHODS

Studying the morpho-functional peculiarities of the lower limb joints in the red squirrel, was done by their stratigraphic dissection follow the muscles action of the jointing extremity, in this mode establishing the type of movement and its peculiarities at the species studied. Then, they resorted to scraping the bone adjacent tissue to the bone, following the length and layout the jointing and muscles processes and extent of the jointing surface of each bone. The peculiarities found were photographed, described and compared with similar structures found in other species, aiming to permanently their interpretation of the basic anatomical principles, in terms of cause and effect.

## 2. RESULTS AND DISSCUTION

The squirrel, compared with the rest of rodents, sacrum vertebrae consists only of two welded together (Hriţcu Valentina *et al.,* Hriţcu Valentina, V. Coţofan*).* First sacral vertebra has the same with as the

lumbar spine and whole body length due to development of the sacrum it looks rectangular wings (Fig. 1). The wings are large, present along the length of the coxal jointing surface which is horizontally oriented (Spătaru Mihaela, C. Spataru).

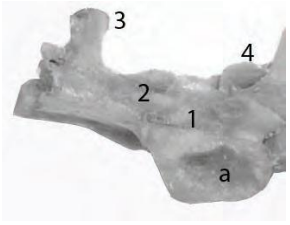


***Fig. 1. The latero- dorsal aspect of the sacrum in red squirrel***
**1- processus articularis caudalis, 2-processus transversus, 3- processus spinosus, 4- procesus mamilaris, a- facies auricularis.**

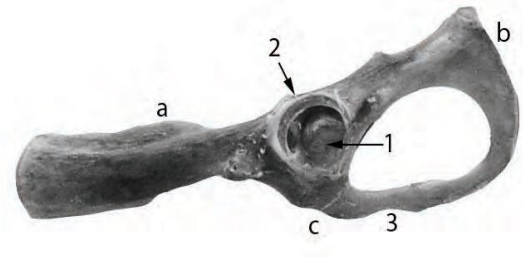


***Fig. 2. Lateral aspect of the coxal bone at red squirrel***
**a-os ilium, b-os ischii, c-os pubis 1-acetabulum, 2- labrum acetabulare, 3-symphysis pelvina**

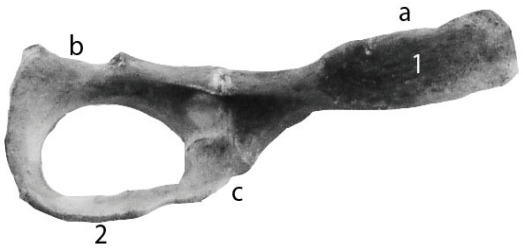


***Fig. 3. The medial aspect of the coxal bone at red squirrel***
**a-os ilium, b-os ischii, c-os pubis 1- Facies auricularis, 2- symphysis pelvina.**

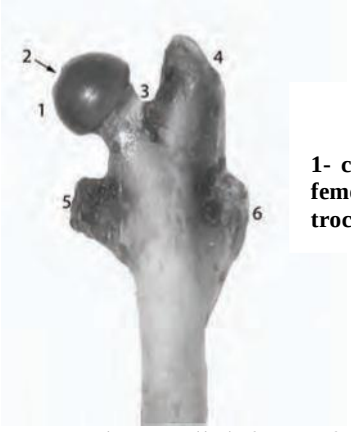


***Fig. 4 The proximal extremity of the femur at red squirrel***
**1- caput ossis femoris, 2- fovea capitis, 3-collum ossis femoris, 4- trochanter major, 5- trochanter minor, 6- trochanter tertius**

On the medial face of the ilium, is a small articular surface for the sacrum uniform appearance, placed till the neck of ilium (Fig. 3).

The coxofemural joint at squirrel is particular through its large articular capsule which extends from the edge of the coxal cavity to the base of the higher throcanter, which allows very large movements (Fig. 2). In addition, capsular ligament in thickened, with three small flaps: cranial, caudal and medial. Cranial fascicle goes below the tuber for the rectus femoris inssertion, its fibers are distributed as a fan in the entire capsule. This limits the joint extension and the abduction. The caudal fascicle is more individualized, appears like a collateral ischio-femoral ligament. Its fibers go from the caudal edge of the cothiloid cavity that corresponds of the ischium to the femoral neck, near the second trochanter. The ventral fascicle is wide, it is inserted on the evident edge that medial form the articular surface of the femoral head and then they lost in the capsular ligament. These restrict the abduction movements. Lack of the capsular muscle is supplied by periarticular muscles, especially the gluteus profundus. In addition, at the squirrel, the obturatorius internus tendon is long, thin and very strong and it opposite to the abduction movement of the hip joint (Fig. 6).

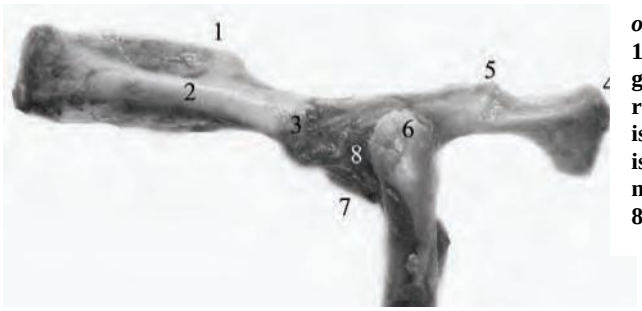


***Fig. 5 The lateral aspect of the hip joint at red squirrel***
**1- Os ilium, 2- linea glutaea, 3-area lateralis m. recti femoris, 4- tuber ischiadicum, 5- spina ischiadica, 6- trochanter major, 7- capsula articularis, 8- m. gluteus profundus**


***Fig. 6 The dorsal aspect of the hip joint at red squirrel***
**1- Ilium, 2- linea glutea, 3- area lateralis m. recti femoris, 4- trochanter major, 5- capsula articularis, 6- m. gluteus profundus, 7, 8- tendo muschii obturatorius internus.**

The femoral head ligament, the round ligament, appears as a long fascicle thickness of 2-3 mm inserted into the femoral head ligament fosse and surface fosse of the coxal cavity (Fig. 4).

The jointing coxal cavity is round, deep and central presents a ligament fosse for femoral head insertion. On the edge of the cavity is inserted a fibro-cartilage round ligament that is thick and about 1-2 mm high that further deepens the articular surface (Fig. 2). The femoral head articular surface is extended to three quarters of a sphere, ligament fosse is centrally located. The femoral neck is long, moving it medially (Fig. 4). The plan of the hip joint is placed medial to the femur and its axial plane, which shows that the weight and power is distributed and supported by the femoral neck and head, making possible much larger movements of abduction and limb abduction, necessary in movement (Rizac V. *et al*).

Distal articulary surfaces of the femur to the squirrel is similar with at the felines, so the femoral trochlea is superficial and large and the condyls are more detaching caudally, outside the axis of the femur, allowing extension and flexion movements extremely large. Above each condyle is one surface for the femoral sesamoid bones. Tibia and fibula at squirrel are independent bones, jointed to each other to both extremities, making together a large tibio-fibular space (Hriţcu Valentiuna, V. Coţofan).

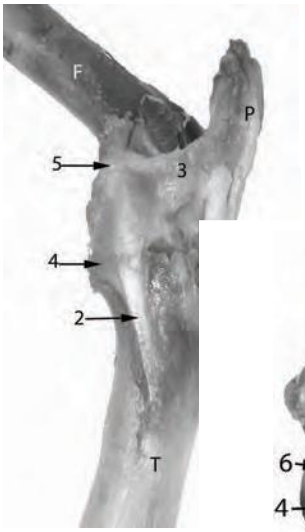


***Fig. 7. Medial aspect of the knee joint at red squirrel***
**F- os femoris, P- patella, T- tibia, 1- lig. Patellae, 2- lig. Collaterale mediale, 3- lig femoropatellare mediale, 4- meniscus medialis, 5- capsula articularis ossa sesamoidea m. gastrocneminii**

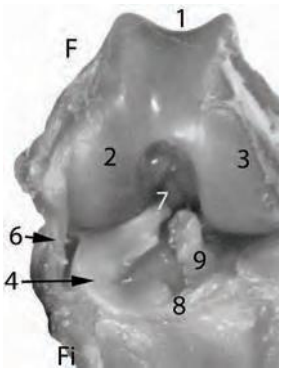


***Fig. 8. The detail of the knee joint at red squirrel***
**1- Trochlea ossis femoris, 2- condylus lateralis, 3- condylus medialis, 4- meniscus lateralis, 5- meniscus medialis, 6- lig. collateralis lateralis, 7- lig meniscofemorale, 8- lig. meniscotibiale laterale, 9- lig cruciatum craniale ,**
**F- os femoris, T- tibia, Fi- fibula**

At the proximal extremity of tibia has the two condylar articular surfaces that easily excavated, being separated by the tibial spine very

reduced in height. At squirrel, the lateral condyle has the articular surface larger caudally, that produces the shank rotation around its own axis with support on the medial condyles: of the femur and the tibia. The medially dragging of tibia is facilitated by the extremely low and the reduction in size of the medial meniscus. The lateral meniscus is longer and thicker and well anchored by menisco-tibial and menisco-femural ligaments than the medial meniscus. In the thickness of each meniscus, to the caudal edge of each, is present one nucleus of ossification (Fig. 8).

The femoro-patella ligaments appear like a fascicle in the capsular ligament thickness that is inserted on the lateral side of the femoral condyles and the lateral edges of the patella (Fig. 7). The collateral ligaments look like pearly white, lateral collateral ligament is more developed and composed of two beams: lig. femuro-tibial and lig. femuro-fibular. Lig. femuro-fibular is thick and durable, slides over the lateral condyle of tibia to insert on the proximal end of the fibula. In the rotation occurs medial tibia and fibula movement. The tibio-fibular joint is a synovial joint, the jointing surfaces being plane and triangular. The jointing capsule is large, allowing the sliding of the articular surfaces (Spătaru C. *et al)*

The tibio-tarso-metatarsal joint at squirrel

The joint between cochlea tibialis and astragalus trochlea obliquity customizes the latero-medial axis joint, the articular surface being lateral completed by fibular malleolus that distal has a jointing surface for calcaneum. Regarding the medio-tarsus joint, the talus is articulated through the three trochleae. The dorsal trochlea is articulated with the tibial cochlea that presents the evident and sharp condyls, obliquely orientated, making large movements of flexion and extension. The caudal trochlea has the deleted condyles separated by a wide and shallow groove. It is articulated with the large calcaneum cochlea, allowing the flexion and extension in relation to the talus. The distal trochlea is like an articular head, caudally being supported by a ventral elongation of the astragalus. It articulates with the proximal joint cavity of tarsal central bone and medial with accessory tarsal bone. Distally, the calcaneum presents an articular surface slightly concave for articulate with a slight convex surface of the cuboideum bone. At the talus-scaphoideum and scapho-cuboideus joints level, both flexion and extension and medial rotation of the foot are produced (Fig. 9).

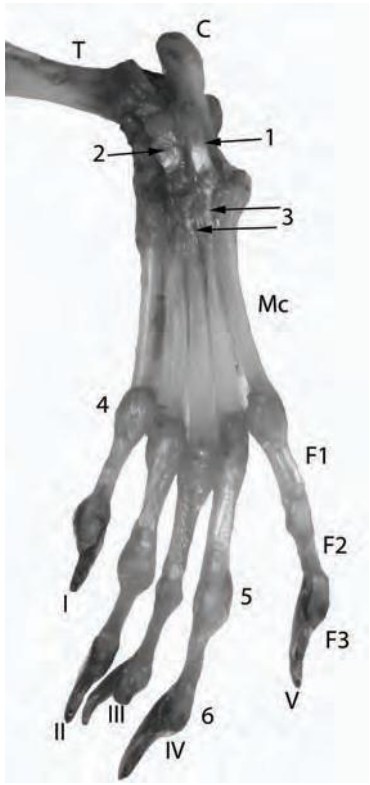


***Fig. 9 The palmary aspect of the foot joints at red squirrel***

**T- tibia, C- calcaneus, Mc-ossa metatarsalia, F1, F2, F3- ossa digitorum pedis**
**I-V- digiti**
**1- Lig. plantare longum, 2- lig. talocentrale interoseum, 3- lig. tarsi plantaris, 4- articulationes metatarsophalangeae, 5, 6- articulationes interphalangeae pedis.**

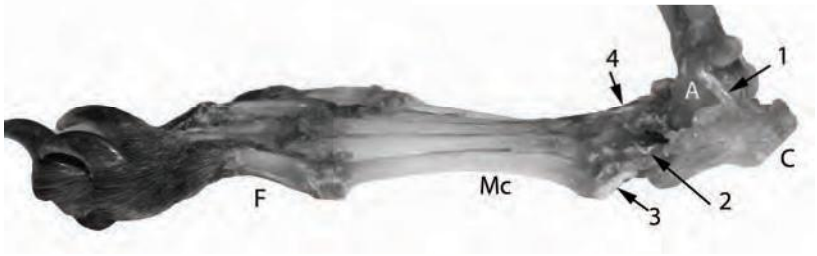


***Fig. 10 The laterally aspect of the foot joins at the red squirrel***

**T- tibia, Fi- fibula, C- calcaneus, A- talus, Mc- ossa metatarsalia, F- ossa digitorum pedis**
**1- Lig. calcaneofibularis, 2- lig. talocalcaneum interosseum, 3- lig. plantare longuum, 4- lig. talometatarsal**

The collateral medial ligament is developed and it is a pearly white inserts on a beam to the malleolus of tibia and first metatarsus ligament tubercle. Collateral lateral ligament is reduced, being substituted by the

fibula-calcaneum and talus-calcaneum. The fibulo-calcaneum ligament fasciculate looks, being inserted on the fibula malleolus and the lateral face of the calcaneum. The strong talus-calcaneum ligament is inserted on the dorsal face of the talus and the lateral face of the calcaneum, close to its distal end. At squirrel, the form and position of the common ligaments of the jointing complex permit the dorso-lateral rotation of the foot (Fig. 10).

## 3. CONCLUSIONS

3.1. Lack of the capsular muscle of hip joint at squirrel is supplied by the periarticular muscles, especially the gluteus profundus and the obturatorius internus tendon that is long, thin and very strong and it opposite to the abduction movement of the hip joint.

3.2. The plan of the hip joint is placed medial to the femur and its axial plane, which shows that the weight and power is distributed and supported by the femoral neck and head, making possible much larger movements of abduction and limb abduction, necessary in movement.

3.3. At squirrel, the lateral condyle of tibia has the articular surface larger caudally, that produces the shank rotation around its own axis with support on the medial condyles: of the femur and the tibia.

3.4. The lateral meniscus is longer and thicker and well anchored by menisco-tibial and menisco-femural ligaments than the medial meniscus, aspect that allows the shank rotation.

3.5. At squirrel, the form and position of the common ligaments of the jointing complex of the heels permit the dorso-lateral rotation of the foot.

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# INFLUENCE OF THE INTENSIVE HUSBANDRY ON INNATE IMMUNITY IN DAIRY COWS

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**Keywords:** intensive farming, bovine, innate immunity

## SUMMARY

The immune system is being considered a very sensitive indicator of stress in all species (Harmon, 1997). This research aimed to monitor the global influence exerted by intensive farming conditions on indicators of innate immunity such as total immune globulins (tIg), circulating immune complexes (CIC) and phagocytosis in milking cows (n=28) aged 5 to 11 years. Sera and blood samples collected on heparine were subjected to zinc sulphate precipitation, polyethylene glycol precipitation and carbon particle inclusion test, respectively. Both tIg and CIC values were red spectrophotometrically after 30 ($\lambda$=475 nm) or 60 ($\lambda$=450 nm) min of incubation, respectively and expressed as conventional units. Phagocytosis was monitored in untreated and an alcoholic *Silybum marianum* extract treated samples, red spectrophotometrically after 0 (t0), 25 (t1) and 50 (t2) min of incubation at 37°C. Optical density readings were converted to a log2 scale and phagocytic index was taken as the negative of the slope of the regression of optical density (log2) (t0-t1, t1-t2). There was an increase in both tIg (51.8±11.2) and CIC (6.80±5.45) levels when compared to physiological values, probably due to a higher bacterial load of the environment and increased stress induced by the raising technology. Spontaneous phagocytosis was negatively influenced by the increase in incubation time (0.01±0.05, during the first period versus -0.13±0.16, during the second period). The *Silybum marianum* extract exerted a positive effect during the second period of incubation (0.02±0.12 versus -0.14±0.10), suggesting the possibility of its use as a phagocytosis stimulating agent. We concluded that intensive farming factors increased the innate humoral immunity and decreased the cell mediated immunity in milking cows.

Dairy cows have long economic lives and the intensive rearing system by its artificial environment asserts physiological adaptation problems which often lead to failure (Onaciu, 2006). There are several stress factors in this type of husbandry, including prolonged or exclusive tied rearing, reduced space allowances, overcrowding of animals, adverse microclimate, early weaning, psychological stress, artificial inseminations and more (Morar *et al.*, 2005). Nowadays, livestock sciences plead for current technologies, with small, medium and large cattle herds, taking into account all the interrelations, achieving an acceptable compromise between biology, physiology and ethology of dairy cows, according to breed, age category and productions and

economic, technical, hygienic and environmental requirements (Man *et al.*, 2002).

This work aimed to monitor the influence of intensive husbandry on innate immunity in dairy cows, in order to estimate the resistance to diseases on a conventional dairy cow farm.

## 1. MATERIAL AND METHODS

**Animals**. Milking cows (n=28) of Romanian spotted breed were subjected to this study. The animals were raised intensively and kept during the cold season in enclosed barns and in the warm season in summer camps, on the pasture. The barns are built of bricks, with plastered walls, provided with lateral windows and 3/3m doors for the access of feeding equipment and two lateral doors for the workers' access. The barn capacity is of 120 cows, animals are tethered in two rows, ordered head-to-head. Lighting is both natural through the lateral windows and artificial through the illumination sources disposed centrally on the barn's ceiling. Lighting level is poor due to dirty windows and small number of dirty electric light bulbs. The ventilation system is only natural, through air inlets and the frontal and lateral doors, when these are open.

Feeding of cows is mechanical using a tractor with trailer, they have float bowl waterers for drinking; drinkers and feeders are manually cleaned by employees. Winter fodder includes hay, produced on the farm's land and stored inside the sheds; grains and succulent fodder (shredded beetroot, silage). In summer the cows' daily forage is green fodder (grazing) supplemented with concentrates (grains).

Manure cleaning is carried out mechanically, using scrapers. Manure is evacuated twice a day, in the morning and in the evening; between evacuations manure is stored in the collecting canal. Bedding materials are straw or sawdust.

Sera were obtained from whole blood, sampled in sterile test tubes containing a procoagulant gel, after separation by centifuging the samples.

**Tests**. Within our experiment the total gamma globulins were quantified by use of a micro variant of the 24%o zinc sulphate precipitation test (Serb reagent) in 96-well, flat bottom plates. 196.7 microliters of reagent were mixed with 3.3 microliters of serum, allowed to incubate at room temperature for 30 min and red spectrophotometrically (Sumal PE 2, Karl Zeiss, Jena) at a wave length

of 475 nm. The obtained results were expressed in Vernes degrees, multiplying the optical density units read by 100.

The levels of circulating immune complexes were obtained by a 4,2% polyethylene glycol (PEG) technique. Aliquots of 193.3 microliters of PEG and borate buffer were each placed in contact with 6.7 microliters of serum in 96-well plates, allowed to incubate for 1 h at room temperature and read at 450 nm wavelength (Sumal PE2, Karl Zeiss, Jena) according to the method described by Ghergariu *et al.* (2000). Total CIC amounts were calculated by substracting the precipitation value given by the borate buffer in contact with the serum and multiplying it 1000 times.

Phagocytic cells engulf inert particles such as carbon due to the defensive capacity of these cells. An *in vitro* carbon clearance assay was used to evaluate phagocytic activity in the experimental batch, adapted from the technique described before (Khokhlova et al., 2004). 1.5 ml of venous blood collected on heparine (50 IU/ml) received 6 µl of the supernatant fraction of India ink (Pelikan AG D-3000, Hanover, Germany) that had been centrifuged at 3000 *g* for 30 min. After mixing, each sample was divided into 3 equal aliquots, of which one untreated, one treated with 70° alcohol and another treated with a *Sylibum marianum* (1.5 microliters/tube) alcoholic extract, respectively. Each variant was incubated at 37°C for 30 min. Volumes of 150 µl of each mixture were taken and added to 2 ml saline after 25 and 50 min of incubation, respectively. These diluted samples were centrifuged at 50 *g* for 4 min and the supernatant was read spectrophotometrically at 535 nm, with the background taken as zero. There was a decrease in absorbency with time as carbon was phagocytosed. Optical density readings were converted to a log2 scale and phagocytic index was taken as the negative of the slope of the regression of optical density (log2) on time (min).

The enhancing/inhibiting effect of an alcoholic *Silybum marianum* extract was monitored, in comparison to spontaneous and 70° alcohol induced phagocytosis. The data were statistically processed by use of Excel program, with mean values, standard deviations and statistical significance of the differences (Student's t test) being calculated.

## 2. RESULTS AND DISCUSSION

The serum level of immune globulins is an indicator of global humoral reactivity. Changes occurring in a direction or another indicate either the enhancement of the immune response or a lack of humoral

immune reactivity, influenced by either external or internal factors (Roitt and Delves, 2001; Zahao, 2006).

One of the main components of the immune respones is represented by antibodies, proteins related from chemical, phylogenetic, functional and antigenic points of view. In 1970, specialists of WHO (World Health Organization) established by consensus that molecules with antibody properties can be grouped in the category of immune globulins, based on the fact that all the substances in this group have immune functions and are comprised in the globulin fraction of the serum.

The microbial agression induces an immune response expressed by gamma globulin secretion with antibody activity and appearance of sensitized cells, agents of the cell-mediated immunity (Turner, 1994; Goldsbi *et al.*, 2001).

Investigations carried out on total immuneglobulin levels (Table 1) of dairy cows kept in intensive husbandry system indicated the lowest values of 30,9 Vernes degrees and the highest is of 65,8 Vernes degrees.

***Table 1***

**Mean values of total serum gamma globulins in dairy cows kept in intensive raising system**

| Sample number | ODU | Vernes degrees |
|---|---|---|
| Mean | 0,518 | 51,8 |
| Standard deviation | 0,112 | 11,2 |

ODU = Optical Density Units

In spite of statistically non significant differences between the reference values for bovine and obtained results for cows, intensive breeding induces in the enhancement of the immunoglobulin synthesis, the recorded mean values being higher.

These findings suggest that the higher microbial load subsequent to the existing technological flow and also the more intense circulation of humans and vehicles within the farm leads to an increased response by the involvement of total Ig pool.

CIC levels provide in many infections a measure of the body's reactivity and of the severity of diseases. In some microbial diseases, the excessive synthesis of CIC can lead to their depositing in various organs with the activation of the complement membrane attack complex and the subsequent destruction of tissue, release of new antigens and the formation of antibodies directed against modified self (Bajaj et al., 1990).

The individual values of the total circulating immune complexes in dairy cows kept in intensive husbandry system (Table 2) varied within a wide range. When compared to physiological levels, there was no statistical significance of the differences.

***Table 2***

**Mean values of the serum circulating immune complexes in dairy cows kept in intensive husbandry system**

| | PEG (ODU) | Buffer (ODU) | CIC (Units) |
|---|---|---|---|
| Mean | 0,05 | 0,04 | 6,80 |
| Standard deviation | 0,01 | 0,01 | 5,45 |

ODU = Optical Density Units

The results obtained for CIC lead to the conclusion that these are influenced by the husbandry system; stress caused by overcrowding of animals and the microclimate and animal care conditions. All of these factors increase the clearance rate of immune complexes and seem to slightly diminish the serum levels of the circulating immune complexes in dairy cows kept in intensive husbandry system.

Phagocytic cells engulf inert particles such as carbon due to the defensive capacity of these cells (Hilton *et al.*, 2002). The reactivity of innate immune cells can be monitored in such a manner and their direct reactive potential can be quantified in the case of microbial aggression or under the influence of stress factors. The phagocytic activity can be positively or negatively influenced by several herbal extracts. In this respect we used the alcoholic extract of *Silybum marianum*.

The results of the phagocytic activity recorded in the group of dairy cows kept in intensive breeding were shown in table 3.

***Table 3***

**The phagocytic activity values during the study period in dairy cows bred in intensive system (nl)**

| | Control sample | | | Variant treated with alcohol | | | Variant treated with *Silybum marianum* | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0’ | nl0'-nl25' | nl25'-nl50' | 0’ | nl0'-nl25' | nl25'-nl50' | 0’ | nl0'-nl25' | nl25'-nl50' |
| Mean | | 0,01 | -0,13 | | -0,06 | 0,001 | | -0,14 | 0,02 |
| SD | | -0,05 | -0,16 | | -0,17 | -0,01 | | -0,10 | -0,12 |

nl = Natural logarithm; SD = Standard deviation

The phagocytic activity in the studied group of intensively raised dairy cows indicated positive values in the untreated control samples

during the first incubation period, while the 70° alcohol and the *Silybum marianum* extract inhibited phagocytosis. Nevertheless, during the second reading period, the stimulating effect of the vegetal extract suggested its potential use in enhancing innate cell mediated reactivity.

## 3. CONCLUSIONS

3.1. Intensive husbandry, with its multiple stressful factors, induces in dairy cows an increase in immune globulin synthesis, as well as in the clearance rate of immune complexes and it seems to slightly diminish the serum levels of the circulating immune complexes.

3.2. Although pahgocytosis was decreased when compared to physiological values, the *Silybum marianum* 70° alcoholic extract seems to be useful in controlling in a positive sense the activity of phagocytic cells.

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# MODULATING POTENTIAL OF CERTAIN VEGETAL EXTRACTS ON ADAPTIVE CELL MEDIATED IMMUNITY IN INTENSIVELY RAISED DAIRY COWS

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**Keywords:** milking cows, intensive raising, adaptive immunity, vegetal extracts

## SUMMARY

The artificial microclimate and intensive raising are factors of influence and disrupt the fragile balance of highly productive animals as milking cows. This study aimed to investigate the potential *in vitro* use of certain vegetal extracts in modulating the specific cell mediated-immunity of milking cows. The protocol included a single blood sampling from the jugular vein of each of 28 Romanian Spotted animals, aged 5 to 11 years, raised on an industrial farm. Blood was collected on heparine (50 IU/ml), mixed with RPMI 1640 culture medium, distributed in 200 µl aliquots in 96-well plates and treated with vegetal extractions, as follows: untreated control sample; phytohaemagglutinin treated variant; 70° alcohol (solvent) treated control; *Silybum marianum* treated variant; *Hippophae rhamnoides* treated variant; *Vaccinium myrtillus* treated variant; *Thymus vulgaris* treated variant; *Aloe vera* treated variant. All variants were performed in duplicate, using 1,5 µl of compound / well. The plates were incubated at 37°C in a 5% CO2 atmosphere for 60 hours. Cell growth was estimated by spectrophotometrical measurement ($\lambda$=610·nm, d=0.5·cm) of the glucose residue in a colorimetric orto-toluidine test. Blast transformation indices were calculated as percentages of the consumption versus the initial glucose concentration of the RPMI 1640 medium. The data were statistically interpreted, by calculating mean values and standard deviations as well as the statistical significance of the differences by use of Student's t test. The results indicated inhibitory effects for all tested extracts when compared to the untreated control (60,28±37,73%), suggesting their negative influence on blast transformation capacity of leukocytes. The *Aloe vera* extract was the only one to stimulate cell growth, when compared to the solvent treated control (57,94±37,19% versus 54,86± 21,71%). Out of the tested extracts none could be used to stimulate adaptive cell mediated immunity in cows raised under intensive farming conditions.

Cattle raising represents "a living industry" which processes agricultural raw materials in animal products with remarkable biologic value. In average breeding conditions a cow can provide the necessary meat for six to eight people and the necessary milk for 10 to 15 people (Morar *et al.*, 2005; Man *et al.*, 2002).

Monitoring the changes of the immune system may provide useful information for dairy cow breeders to ensure optimal rearing and welfare conditions for their animals (Onaciu, 2006). Researches in order to establish the effects of various herbal extracts on the immune system and

farm animal productivity are being done within the framework of detailed experiments for the discovery, characterization and use of some plant compounds containing immune modulating substances (Liu and Ng, 2000; Vanmiert, 1991).

Stressful factors strongly interact with the immune system and intensive farming provides numerous examples of such factors. Therefore, this study intended to monitor the influence of intensive raising on milking cows' adaptive cell-mediated immunity and the potential of certain vegetal extracts from the specific Romanian wild flora to enhance this *in vitro* response.

## 1. MATERIAL AND METHODS

Twenty-eight Romanian spotted breed milking cows, raisen under intensive farming conditions, were assessed for their spontaneous and mitogen induced blastogenic activity. The animals were mainly kept indoors during the winter and on the pasture during the summer. The barn capacity was of 120 cows, animals are tethered in two rows, ordered head-to-head. The ventilation system is only natural, through air inlets and the frontal and lateral doors, when these are open. Feeding of cows was mechanical using a tractor with trailer, they have float bowl waterers for drinking; drinkers and feeders are manually cleaned by employees. Manure cleaning was carried out mechanically, using scrapers. Manure was evacuated twice a day, in the morning and in the evening. Straw or sawdust was used as bedding.

Whole blood samples were collected by puncturing the jugular vein, on heparine (50 IU/ml). Cell growth was quantified by means of the glucose consumption technique. Part of the blood sample (640·µl) was diluted with four times the amount of RPMI 1640. The mixture was distributed in a sterile 96-wellplate (200·µl per well). Eight variants were tested once for each individual animal, namely (1) untreated control culture, (2) phytohaemagglutinin-M (PHA) (1·µl per well) treated culture, (3) alcohol, (4–8) alcoholic extracts of milk thistle (*Silybum marianum*), common sea-buckthorn (*Hippophae rhamnoides*), bilberry (*Vaccinium myrtillus*), common thyme (*Thymus vulgaris*) and medicinal aloe (*Aloe vera*) (1.5·µl per well) treated cultures. The quantities of both PHA and antigens were established when using the same technique during preliminary studies as being the most effective *in vitro* for the tested species. The cultures were incubated for 60·h at 37.5°C and 5% CO2. Glucose concentrations were measured in the initial medium and in all variants at the end of the incubation period, using a standard

(100·mg·dl–1) glucose solution, by means of an orto-toluidine colorimetric test. To do this, 12.5·ml of the cultural supernatant were transferred to 0.5·ml of orto-toluidine reagent, boiled for 8·min, cooled suddenly in cold water and read in a spectrophotometer at 610·nm wavelength (Sumal PE2, Karl Zeiss, Jena, Germany), using the reagent as a blank. The transformation index (TI) was calculated as follows: TI%=[(MG–SG)/MG]´100, where TI=blast transformation index, MG=glucose concentration in the initial culture medium and SG=glucose concentration in the sample after incubation.

The results were subjected to statistical calculations by means of Excel program, mean values, standard deviations and the statistical significance of the differences being established.

## 2. RESULTS AND DISCUSSION

The proliferative response of lymphocytes can be assessed by the *in vitro* blast transformation test (BTT), which provides data on both spontaneous of mitogen induced reactivity of specific immune cells. Similarly, this method was used to assess the response of lymphocytes or leukocytes in the whole blood against different bacterial or viral antigens (Spinu *et al.*, 1995, b, c).

Experiments indicated that there are several compounds obtained from plants, bacteria or even cells, secretions or toxins, able to induce blast trsnformation even if lymphocytes or leukocytes had no previous contact with these (Devasagayam and Sainis, 2002; Dewick, 1997).

The test is suitable for the investigation of several herbal extracts' effect, when solvents used to obtain the active substances are miscible with a water soluble medium used for the culture of immune cells (Davison, 2003; Spinu *et al.*, 1996; Spinu *et al.*, 1995, d).

The results obtained for the blastization in dairy cows raised unde intensive conditions (Table 1) indicated a lower mean value of the stimulation index in the control (untreated) variant when compared to the mean values obtained for the alcohol and plant extract treated variants. The classical mitogen acted less stimulating than expected, while the alcohol anfd the various extracts were inhibiting the cell growth. The very high standard deviations suggested a high variability of the individual values under conventional raising conditions of milking cows.

***Table 1***

**Mean values of the stimulation indices obtained in the blastic transformation test in the group of intensively bred dairy cows (%)**

| | C | PHA | Alc | Sm | Hr | Vm | Tv | Av |
|---|---|---|---|---|---|---|---|---|
| Mean | 60,28 | 60,09 | 54,86 | 50,56 | 48,79 | 52,52 | 46,92 | 57,94 |
| SD | 87,73 | 89,84 | 91,71 | 90,18 | 89,66 | 87,15 | 87,78 | 87,19 |

C = control sample, untreated; PHA= variant treated with phytohemagglutinin; Alc = variant treated with 70° alcohol; Sm = variant treated with *Silybum marianum*; Hr = variant treated with *Hippophae rhamnoides*; Vm = variant treated with *Vaccinium myrtillus*; Tv = variant treated with *Thymus vulgaris*; Av = variant treated with *Aloe vera*; SD = standard deviation

Among the alcoholic plant extracts used *in vitro*, the thyme (*Thymus vulgaris*) extract induced the lowest mean value of the stimulation index. Excepting the medicinal aloe (*Aloe vera*) extract, when compared to the alcohol, but not to the untreated control, the results indicated inhibitory effects for all tested extracts, suggesting their negative influence on blast transformation capacity of leukocytes. No statistically significant differences were obtained versus control or between the vegetal extracts' treated variants.

The lack of *in vitro* reactivity of the leukocytes to the polyclonal mitogen were in contradiction with scientific data indicating a strong stimulating effect of such treatments, especially on T lymphocytes (Fudge, 2000; Tizard, 1996). It is possible than this lack of response was generated by stress, induced by a wide range of stress factors: environmental, behavioral, nutritional, infectious, parasitical and more, with repercussions on the cellular effectors affecting the cell membrane of lymphocytes and blocking the receptors or deviating the transduction signals. A similar mechanism could be involved in the mild responses to vegetal extracts out of which some were indicated as stimulating for animal immune cells (Peretianu and Saragea, 1998; Roitt, 2001).

## 3. CONCLUSIONS

3.1. The *in vitro* blast transformation test, used as a tool to monitor the effects of certain vegetal extracts on adaptive cell mediated immunity indicated the suppressive effects of all tested extracts when compared to the untreated control, suggesting their negative influence on blast transformation capacity of leukocytes.

3.2. Out of the tested extracts none could be used to stimulate adaptive cell mediated immunity in dairy cows raised under intensive farming conditions.

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# THE STUDY OF ANTIBACTERIAL EFFECT OF XCUO (100-X) [55$B_2O_3$ 45ZNO] VITREOUS SYSTEM

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**Keywords:** glass, copper, zinc, antibacterial.

**SUMMARY**

This paper studied the antibacterial effect of the xCuO (100-x) [55$B_2O_3$45ZnO] vitreous system for $0 \leq x \leq 15$ mol%, by the method of dilution in simple broth, the degree inhibition degree being measured by spectrophotometry.
The prepared system presents an inhibitory effect over the *E coli* while the *Micrococcus lysodeicticus* proves to be little sensitive in the interaction with this agent. For the *E coli*, the inhibition is due more to ZnO than to the CuO, the minimal optical density measured being four times smaller than the one of the control sample.

The investigated compounds are part of $B_2O_3$ vitreous structure ones to which ZnO was added in a adequate proportion in order to form the vitreous or crystalline structure (Schubert et al., 2003). The boron oxide is a classic former of vitreous network, when it is melt and undercooled. (Wright et al., 1995). It produces, according to the melting temperature and the oxides it is mixed with, functional groups based on boron (metaborate, ortoborate, pyroborate etc.) containing [$BO_3$] and [$BO_4$] units (Kamitsos et al., 1987). The ratio of these units reflects in the physical properties and in the behavior of the compound in different biological fluids.

The zinc oxide (ZnO) is widely used in the pharmaceutical industry for preparing antibacterial creams (Hernández-Sierra et al., 2008; Husheng et al., 2008; Stefan et al., 2010). The zinc oxide can form a alone a glass network but, at the same time it can be a network modifier particularly when it is melted with $B_2O_3$[Motke, 2002 #444].

Along with the two compounds, the copper oxide has been used from ancient times for manufacturing working tools, being well known the fact that it has a proven antibacterial effect (Hernández-Sierra et al., 2008). By melting basic oxides in oxide glass, both $Cu^+$ and $Cu^{2+}$ are obtained meaning both the state of valence without antibacterial effect and the valence with this effect.

In this paper we investigated the xCuO(100-x) [$55B_2O_3$ 45ZnO] vitreous system for $0 \leq x \leq 15$ in order to be used in medical applications. By the method of serial dilutions E Coli and Micrococcus stems were investigated.

## 1. MATERILAS AND METHODS

Oxide glass belonging to xCuO (100-x) [$55B_2O_3$45ZnO] vitreous system with $0 \leq x \leq 15$ has been obtained from CuO, $H_3BO_3$, ZnO of reagent grade purity. Mixtures were melted in air at 1200 °C, sintered corundum crucibles, and maintained 15 min. at this temperature.

Molted pieces were broken in a agate mortar and crushed into fine powder. Powders thus obtained were passed through the site with pore diameters of 75 μm to obtain material with controlled grain.

The antibacterial effect of the vitreous compounds was tested in simple broth, agains *E. coli* and *Micrococcus lysodeicticus* field isolates. Thus, in two series of tubes containing 5 ml of broth, the tested compounds were added in amounts of 50 mg of compound, inseminated with 35 microliters of a 24 h culture of either *E. coli* or *M. lysodeicticus*. The tubes were incubated for 24 h at 37°C, then inactivated with 1 ml of a 1% formaline solution/tube for 1 h and red spectrophotometrically at a wavelenght of 535 nm, d = 0.5 cm, against the simple broth control. For each series of tubes, unsupplemented simple broth was inseminated to serve as control. Optical densities were recorded for each treated culture.

## 2. RESULTS AND DISCUSSION

In this paper xCuO(100-x)[$55B_2O_3$ 45ZnO] system was investigated by the method of serial dilutions in order to determine its inhibitory capacity in the case of two gram positive and gram negative bacterial stems, and in order to be able to differentiate the effect in the case of the tow types of stems. The optical density (OD) of the fluid where the microorganisms were developed was considered as an index of the bacterial development. The oxide samples doped with copper oxide have an antibacterial effect (Pickup et al., 2006), as a result of the modification in the valence state of the used oxide due to the melting temperature of the powders and the sub-melting speed. At the same time the zinc oxide is used in many medical applications with a practically proven antibacterial effect and it is supposed to have an inhibiting effect in the case of investigated stems.

The xCuO (100-x)[55$B_2O_3$ 45ZnO] system contains both mentioned oxides and has inhibiting effect for *E. coli* (Fig. 1). All investigated samples have a lower turbidity that the turbidity of the control sample.

**Fig. 1 The optical density of the vs. x for *E.coli***

In the control sample the bacteria is inoculated without adding any other compound, in a characteristic nutritional environment and this represents the medium turbidity of free growth of the bacteria.

The independence of the samples turbidity on CuO concentration in the vitreous matrix as well as small differences of optical density between $B_2O_3$ ZnO glass matrix and transitional metal (CuO) doped matrix is an index of the fact that the antibacterial effect is due to the zinc in the matrix, the latter behaving as an antibacterial agent, the action mechanism being poorly known by now. (Hernández-Sierra et al., 2008). At the same time, the constant of the values of the optical density along to the investigated compositional interval denotes the chemical stability of the compounds in the biological environment.

Even if the copper and zinc have a proven antibacterial effect, in the case of Micrococcum bacteria (Fig.2) these don't have an inhibiting effect (Kim et al., 1998; Pickup et al., 2006; Stefan et al., 2010).

**Fig. 2 The optical density dependence on copper molar content in vitreous system x CuO (100-x) [$55B_2O_3$ 45ZnO]**

The values of optical densities for each copper oxide concentration of the samples are close to the optical density of the witness, the spectral domain where is the optical density of the witness being [0.01; 0.08]. The sample x = 0, with no CuO has a slight inhibiting effect. In the compositional domain x $\in$ [ 1,7 ] mol%, the bacterial increase is stimulated by the presence of the compounds. The only antibacterial effect appears for x = 10 mol% CuO. Because the turbidity of the samples don't depend on the concentration of copper oxide, the interaction as a result of the release in the tomato juice of the ions from the surface of the oxide powder and the compounds present a chemical stability in the liquid where they were immersed, being able to be used in implants or as powders and tinctures at the surface of the skin.

## 3. CONCLUSIONS

3.1 Homogenous vitreous structures of x CuO (100-x)[$55B_2O_3$ 45ZnO] systems were obtained for $0 \leq x \leq 15$ through the method of melt undercooling.

3.2 For the E coli stem the compounds have an inhibiting effect especially due to the zinc oxide compound.

3.3 The investigated samples do not exibit an inhibiting effect over the Micrococcus strain.

3.4 The vitreous samples of CuO(100-x)[$55B_2O_3$45ZnO] system have highly chemical durability in the investigated compositional range.

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# THE STUDY OF ANTIBACTERIAL EFFECT OF $XY_2O_3$ (100-X)[ $55B_2O_3$ 45ZNO] VITREOUS SYSTEM

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**Keywords:** vitreous, yttrium, silver, antibacterial.

**SUMMARY**

The present paper investigated the antibacterial effect of the $xY_2O_3(100-x)$ [$55B_2O_3$ 45ZnO] system for $0 \leq x \leq 11$ on E. Coli strain by comparing with $AgNO_3$ containing PMMA (polymethyl metacrylate). Vitreous materials have been obtained by undercooled method and crushed in powder with certain size.
A significant decrease of optical density from control sample ones, was recorded using spectroscopical method, when introducing the vitreous system in nutritive medium togheter with *E. Coli* strain.
The investigated composite material shows no inhibitive behavior for all tested samples, optical density (OD) values for different siver concentration concentrations, being close to those achieved for the control sample.

$B_2O_3$ oxide glass were investigated both from the structural point of view and from the viewpoint of their application in the medical field, namely in the field of implants, they being an alternative to $P_2O_5$ or $SiO_2$ systems (Kashchieva et al., 2005; Manupriya et al., 2009). These can be prepared in $B_2O_3$-MeO binary compounds (where M is an alkaline or alkaline-earth metal but also some transitional metals), obtaining oxide glass with different chemical durability and applicability in different medical areas. (Kamitsos et al., 1986; Saranti et al., 2006). Zinc oxide is an antibacterial agent and its introduction in oxide matrixes along with boron makes it applicable on a large scale. (Hernández-Sierra et al., 2008). The antibacterial properties of an implantable material increase the chance of it being accepted by the body, by fighting against infection at the place of implant. (Hench, 2006).

Without being known the action mechanism of silver in different types of infections or which is the best or the most efficient chemical combination, the silver ion ($Ag^+$) or the metallic ion is used in order to destroy bacteria. This was incorporated in different polymeric sub-layers (Damm et al., 2008) in order to be used for long time.

In this paper we investigated the $xY_2O_3(100-x)[55B_2O_345ZnO]$ oxide system prepared by undercooling method, comparing its biological effects with the composite material made of PMMA in which $AgNO_3$ was included. The study aimed at the measurement of the antibacterial effect of the investigated system comparing with the system $AgNO_3$ using the bacterial development method in broth.

## 1. MATERIALS AND METHODS

Oxide glass belonging $xY_2O_3$ $(100-x)[55B_2O_345ZnO]$ system for $0 \leq x \leq 10$ have been obtained from $Y_2O_3$, $H_3BO_3$, ZnO of reagent grade purity. Mixtures were melted in air at 1200 °C, sintered corundum crucibles, and maintained 15 min. at this temperature.

Molted pieces were broken in a agate mortar and crushed into fine powder. Powders thus obtained were passed through the site with pore diameters of 75 μm to obtain material with controlled grain.

The investigated composite materials were prepared using commercial PMMA (polymethyl metacrylate ) based cements as starting material (Biomecanica Ind. Brasil), having the following composition: liquid- methylmethacrylate (monomer) 84.4%, butylmethacrylate 13.2%, N:N dimethyl p- toluidine 2.4%, hidroquinone 20 ppm; powder - methylmethacrylate (copolymer) 87.3%, polymethyl metacrylate 2.7 %, barium sulphate 10%. As antimicrobial agent, $Ag\,NO_3$ was incorporated with respect to the total powder amount in a concentration ranging from 0.5% to 10 % w/w.

The antibacterial effect of the invesigated compounds was tested in simple broth, agains *E. coli* field isolates. Thus, in two series of tubes containing 5 ml of broth, the tested compounds were added in amounts of 50 mg of compound, inseminated with 35 microliters of a 24 h culture of either *E. coli.* The tubes were incubated for 24 h at 37°C, then inactivated with 1 ml of a 1% formaline solution/tube for 1 h and red spectrophotometrically at a wavelenght of 535 nm, d=0.5 cm, against the simple broth control. For each series of tubes, unsupplemented simple broth was inseminated to serve as control. Optical densities were recorded for each treated culture.

## 2. RESULTS AND DISCUSSION

In this paper the capacity of bacterial inhibition of $xY_2O_3(100-x)$ $[55B_2O_3\ 45ZnO]$ system by the method of serial dilutions for *E. Coli*

was investigated. The optical density (OD) of the nutritional environment where the microorganisms developed was considered an index of the bacterial development.

The investigated system induces an inhibition effect to the *E. Coli* stem (Fig. 1) the lowest optical density value resulting in the case of x=3 mol%.

**Fig. 1 The optical density of the broth vs. x from $xY_2O_3$ (100-x)[$55B_2O_3$ 45ZnO]**

The values of optical density vary in the interval [0.049;0.08]. As it was shown (Schubert et al., 2003) the properties of $B_2O_3$·ZnO vitreous materials show structural variability without a direct connection to the relation between its two components, but dictated by the concentration of the third component; in this case $Y_2O_3$. In such a way that we expect to find a different dissolution rate, and also a antibacterial effect, not necessarily dependent on the cationic concentration.

In order to test the behavior of polymer compounds in real biological environments we prepared PMMA where we included a known silver content.

The compounds don't have antibacterial effect in spite of the high level of $AgNO_3$ in the polymer. In the compositional field we investigated, the action of the compounds containing yttrium oxide is much more intense than the action recorded the samples containing silver.

Silver distributed uniformly in the polymeric sub layer and consequently in its surface that is why the contact with bacterial development was direct and consistent. .

**Fig. 2 Optical density for *E. coli* vs. AgNO3 concentration in PMMA**

The values of optical densitiy of the samples where composite material was introduced (PMMA and $AgNO_3$), are close to the values obtained in the case of the control sample without compound and the effect is negligible, the sensibility of this stem being very low under the action of silver in the samples. The optical density records values in the range [0.047 and 0.313] independently to $AgNO_3$ concentration in samples.

## 3. CONCLUSIONS

3.1 Homogenous vitreous structures of the $xY_2O_3$ $(100-x)[55B_2O_345ZnO]$ systems were obtained for $0 \leq x \leq 10$ through undercooling the method.

3.2 The investigated vitreous samples have an inhibitory effect over *E. Coli* independently to the soil oxide concentration$Y_2O_3$, a maximal value being for x=3 mol%.

3.3 The investigated composite material PMMA (polymethyl metacrylate) homogeneously mixed with $AgNO_3$ doesn't have antibacterial effect.

## ACKNOWLEDEGEMENTS

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# STUDIES REGARDING SPERM MORPHOMETRY IN DOMESTIC ANIMALS WITH ECONOMIC PURPOSES

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**Key-words:** morphometry, sperm, cryopreservation, morphology assessment.

**SUMMARY**

Morphological type classification of spermatozoa is an important component of the modern semen evaluation; however, current methods of analysis are subjective and highly variable between technicians. Artificial insemination using cryogenic preserved semen is a common management tool of the contemporary livestock producer. However, cryopreservation is detrimental to sperm function and fertility, killing some 50% of the spermatozoa during the process. Prediction of cryopreservation damage from pre freeze samples remains elusive. To reduce the subjectivity and thus variability of sperm morphology assessment, computer automated sperm head morphology analysis (ASMA) has been developed. Previous studies have shown the importance of standardizing ASMA procedures to optimize accuracy. Measurements provided by the ASMA system show that uniform results are acquired among different observers.

The goal of the contemporary breeder is to maximize the efficiency of production, whether it is meat or milk. Reproductive efficiency is a major component of this objective. With the use of cryogenic preserved semen and artificial insemination, male fertility is a large factor in herd reproduction. General semen characteristics such as volume, sperm concentration, sperm motility and sperm morphology characteristics are classic methods of assessing fertility. For over 50 years, scientists have attempted to correlate normal sperm morphology with male fertility. Confounding reports as to this correlation may be due to the variability associated with the methods used to determine normal morphology. Due to subjectivity and variability, development of objective and consistent analysis methods receive considerable attention. One subjective method is computer assisted sperm head morph metric analysis (ASMA). Precise and objective sperm head size and shape can be quantified using ASMA. The objective of this dissertation was to present important results regarding sperm morphometry in boars and bulls.

## 1. MATERIAL AND METHOD

The research in the present paper was carried out on boar and bull semen.

The studied animals are the ones in the top of the breeding pyramid, being tested by the animal individual model B.L.U.P. (Best Linear Unbiased Prediction). Each animal was branded conformingly the methodology stipulated by A.N.A.R.Z. (Animal breeding and reproduction national agency) and A.N.S.V. (Sanitary veterinary national agency).

There were setting off male groups depending on age and breed.

Following every sperm collection there were stained samples using the eosin-nigrosin method. Using the Image Analysis System there were studied 160 random images of the samples with the aid of a specialized soft. For the special sperm files there were determined the indices:

-the total length of spermatozoa;

-the head length;

-the head width;

-the tail length;

The analyzed data were statistically expressed, being recorded the main population parameters: the average, its error, the standard deviation, the coefficient of variability. There were also established the absolute and relative differences among the different categories of age, breed or collection rhythm. The minimum number of spermatozoa required for analysis of sperm head dimensions was found to be about 60 spermatozoa per sample.

There were also calculated the phenotypic correlation values among the analyzed features, after these there were recorded the conclusions.

## 2. RESULTS AND DISCUSSIONS

Artificial insemination using cryopreserved semen is a common management tool of the contemporary livestock producer. However, cryopreservation is detrimental to sperm function and fertility, killing some 50% of the spermatozoa during the process. Prediction of cryopreservation damage from pre freeze samples remains elusive. Computer-automated sperm head morphometry was used in this study to determine the effects of cryopreservation on bovine sperm head morphometry.

Parallel by age, it is noticed the fact that the total length of spermatozoa, was superior in the young boars where the recorded

average value was 51,14±0,48 microns, while in the adult boars this was of 50,76±0,81 microns, the difference being of only 0,38 microns.

Regarding the category of age, it is noticed in the head length of spermatozoa a little difference in favor of the adult boars. Thus, the head length has an average value of 9, 43±0, 09 microns, that means lower with 0, 09 microns against young boars, denoting a non significant difference.

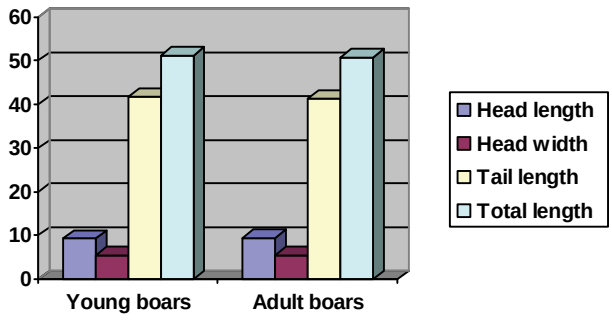
Depending on the category of age too, the length of sperm tail recorded the average 41,80±0,53 microns in young boars and 41,25±0,83 microns in adult boars, the difference of 0,55 microns being non significant.

Regarding to the head width of spermatozoa depending on the category of age we noticed a higher value in adult boars (5, 55±0, 15 microns), against young boars (5, 47±0, 16 microns), but the difference of 0, 08 microns is not significant.

***Table 1***

**The analyzed parameters in boars depending on age**

| Specification | | Age category | | Relative difference |
|---|---|---|---|---|
| | | Young boars | Adult boars | |
| Morphmetric features | Head length | 9,34 | 9,43 | 0,09 |
| | Head width | 5,47 | 5,55 | 0,08 |
| | Tail length | 41,80 | 41,25 | 0,55 |
| | Total length | 51,14 | 50,76 | 0,38 |




**Graph 1. The analyzed parameters in boars depending on age**

In bulls, there were analyzed the same parameters. The average length of the sperm head in young bulls was 8,97 ± 0,87μ, but in the adult bulls was 9,71 ± 0,04μ; the difference between these two groups of

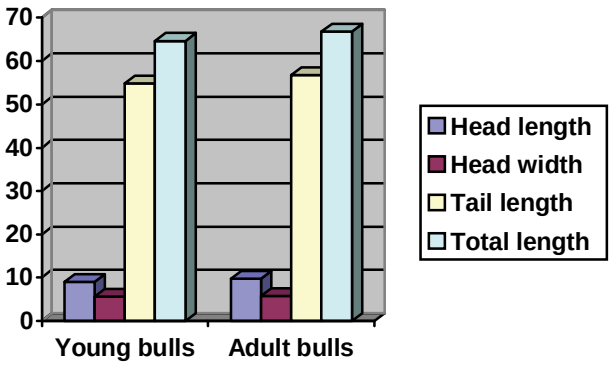
reproducers was 0,74μ, proving a superiority of the adult category of 8,24%. The mean values of the head width was 5, 58 ± 0, 32μ in young bulls and 5, 70 ± 0, 82μ in adult bulls; the recorded difference was low, only 0, 12μ, the adult bulls being superior.

The tail length of the spermatozoa was also higher in adult bulls comparatively the young ones. The recorded value was 56, 75 ± 0, 86μ, respectively 54, 91 ± 1, 45μ, this superiority representing 3, 36%. Also, the total length of the spermatozoa was superior in adult bulls. The total length was 66, 72 ± 0, 82μ in adults and 64, 62 ± 1, 44μ in youth; the absolute difference was 2, 10μ, and the relative one 3, 25 %.

***Table 2***

**The analyzed parameters in bulls depending on age**

| Specification | | Age category | | Relative difference |
|---|---|---|---|---|
| | | Young bulls | Adult bulls | |
| Morphmetric features | Head length | 8,97 | 9,71 | 8,24 |
| | Head width | 5,58 | 5,70 | 2,15 |
| | Tail length | 54,91 | 56,75 | 3,36 |
| | Total length | 64,62 | 66,72 | 3,25 |




**Graph 2. The analyzed parameters in bulls depending on age**

In the paper "Computer automated morph metric analysis of bull sperm heads", G. G. Gravance, R. Vishwanath, C. Pitt and P. J. Casey, found that the mean morph metric measurements for all bulls were the area (27.30μM), perimeter (25.36μM), length (8.65μM), width (4.40μM) and width/length (0.50).

Within the analyses, coefficients of variation ranged from 3.45% for length to 8.52% for area. The ASMA system correctly digitized sperm heads 97% of the time. Results of this study indicate that bull sperm heads can be accurately analyzed using current standard procedures of ASMA technology.

A. Boersma, J. Braun and R.Stolla, publishing the study "Influence of Random Factors and Two Different Staining Procedures on Computer-assisted Sperm Head Morphometry in Bulls" said that the mean spermatozoa head measurements across all slides for area, length and width were 40.49 $\mu m^2$, 9.70 μm and 5.30 μm, respectively.

H. B. Ciftci and U. Zulkadir, in the paper "The correlation between sperm head dimensions and mitochondrial helix length recorded that sperm head length, head width, elongation, ellipticity and mitochondrial length were measured as 14.25±0.17, 7.27±0.23, 0.32±0.09, 1.98±0.18 and 20.30±0.15, respectively.

Comparisons of results from different studies should consider the influence of random and experimental factors to avoid misinterpretation.

## 3. C0NCLUSIONS

3.1. The analyzed parameters in boars depending on age reveal the fact that the adult boars recorded higher values regarding head length and width with low relative differences, 0, 09 in head length and 0, 08 in head width.

3.2. The tail length of analyzed boar sperm recorded the highest value in young boars with a relative difference of 0,55 and implicite the total length of boar spermatozoa.

3.3. The analyzed parameters in bulls depending on age reveal the fact that in adult males the morphometric parameters recorded all the highest values comparatively the young males.

3.4. The results recorded by other different studies show almost the same values, comparisons should consider the influence of random and experimental factors to avoid misinterpretation.

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# COMPARATIVE EVALUATION THROUGH BIOFEEDABACK INVESTIGATION REGARDING RISK FACTORS IN PROSTATE DISEASE FOR HUMAN AND CANINE PATIENTS

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**Key words:** biofeedback, prostate disease, risk factors

## SUMMARY

The present work has followed the identification and corroboration of data, coming from patients with prostate disease, between well established clinical investigation (physical examination, ultrasound) and biofeedback evaluation.
The data obtained using the electrophysiological biofeedback investigation has been compared both in human patients and patients from the canine species which had been previously diagnosed with inflammatory and degenerative disease of the prostate tissue.
Risk factors have been obtained and shown in graphics, and are similar for the two species in regards to: environmental influence, food intake, water pH, radiation exposure, a number of pathogen agents, previous traumas, low immunity level, inflammation sensitivity and incomplete oxidation.
The investigations that have been made are pursuing the corroboration of data pertaining to risk factors for the purpose of the prophylaxis of these conditions on both canine and human and could eventually represent the basis of pilot studies done on canines for the purpose of perfecting treatment and diagnose in human medicine.

In this article we followed 50 cases of patients, 25 human males and 25 canine males that were tested through biofeedback investigation. The main risk factors that occur in patients with prostate disease were identified. The diagnosis for prostate disease was established by clinical investigations, like physical examination and ultrasound, and was confirmed using the electrophysiological biofeedback investigation. We wanted to present the main risk factors that can be found in all patients with prostate lesions.

Our goal was to identify and follow those main risk factors in subjects that have a genetical, age or enviromental disposition to have a prostate related disease, so we can use this knowledge in prophylaxis.

## 1. MATERIAL AND METHOD

In order to conduct this study we used the QXCI dispositive, the harness for the body, legs and the pads for the animals with a greater sensibility, which couldn't be handled. Also, the software on the laptop

was used to translate the electromagnetic impulses into a more specific language.

The electrophysiologic biofeedback investigation analises the whole body through low intensity, uninvasive electro-magnetic pulses using five sensors attached to the subject. The places to attach those sensors are different from one species to another and from one stature or breed to another. In humans the sensors are attached on the forehead, wrists and ankles. For big dogs, we can apply the forehead sensor from humans, by attaching it around the dog's neck, and the other ones on the distal extremities of his four legs. In small dogs, we can use only the sensor for the forehead, but attach it around the dog's thorax.

Biofeedback investigation takes only 15 minutes in order to get information that indicates if there are electrical unbalances in injured cells, and by comparison with known normal electrical values, to determine the nature of the injury or the changes that took place. After this general view of the body, this study concentrated on the risk of disease factors' graphical display.

## 2. RESULTS AND DISCUSSIONS

From the 50 cases we studied, there will be displayed here three cases of male dogs and three cases of human males. Each of the patients went through a protocol, starting with calibration and then testing, in order to confirm on the electrical level, the cellular lesions clinically observed in the prostatic tissue. The lesions were hypertrophy, inflamation, hyperplasia and degeneration, like adenoma or adenocarcinoma. Each patient then got a chart for his major risk factors that could lead to or aggravate his disease.

T.R., a human male, age 54, came to us with an inchoate hypertrophy of his prostate. On his risk factors chart, we observed three of the most frequent factors statistically obtained from our 50 patients' base. These were stress, trauma and inflammation. While the first two were emotion related, the third suggested a physical predisposition. All these factors were contributing to the disease, preventing the ability of the body to maintain its balance. The fact that those risks were situated in the superior extremity of the chart showed that the patient's body was still fighting them.

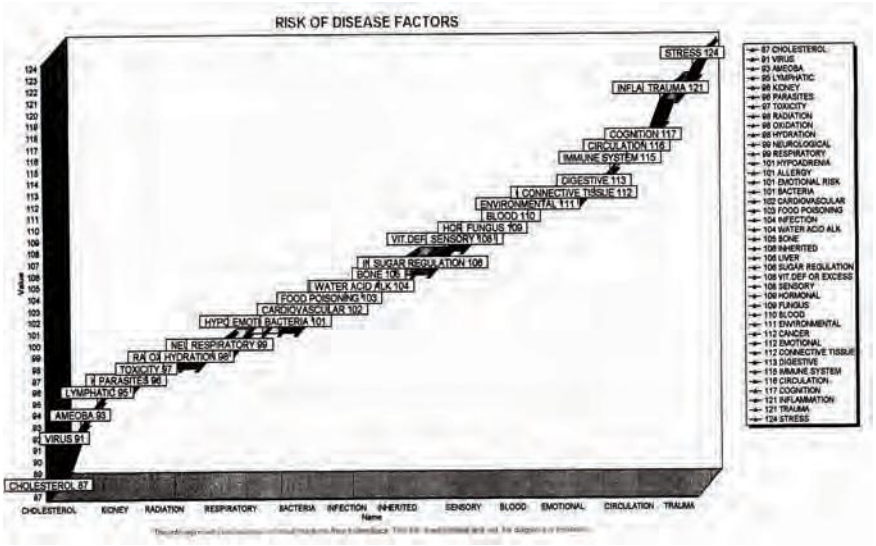


**Fig. 1. Risk of Disease Factors – T.R.**

M.E., a human male, age 51, came with a prostate adenocarcinoma. The risk factors' chart showed six of the characteristic elements in prostate diseases. These were: inflammation, infection, virus, radiation, cancer and stress. All these values were positioned in the inferior extremity of the chart, which suggests that the body had no more strenght to fight them and maintain its internal balance.

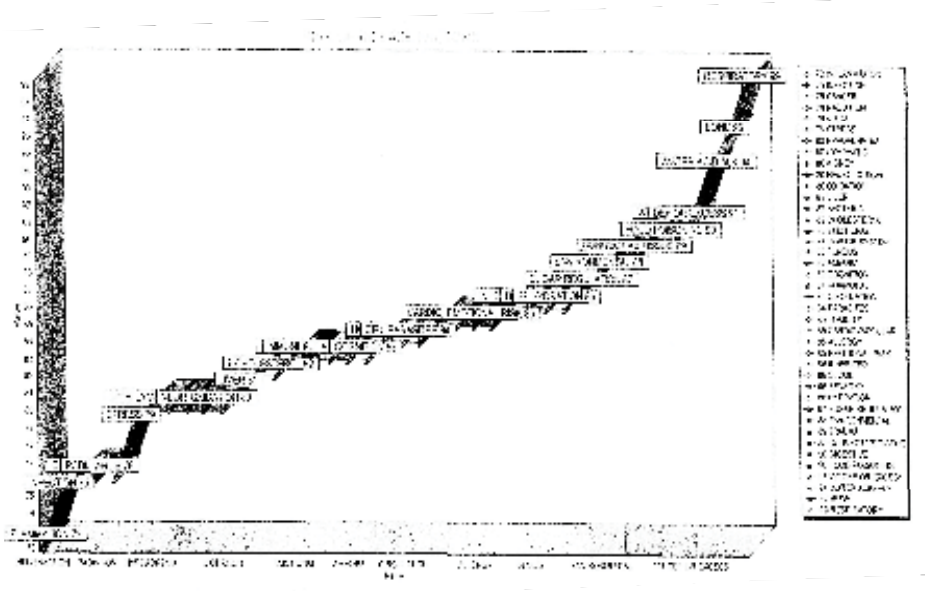

**Fig.2. Risk of Disease Factors – M.E.**

E.D. human male, age 63, was diagnosed with prostate adenoma. His risk factors' chart showed, in the inferior extremity: inflammation, hypoadrenia and trauma. In the superior extremity of the chart he had: low immunity and water pH unbalance. This means that E.D. had a descending vitality, being an easy target for a worse diagnosis.

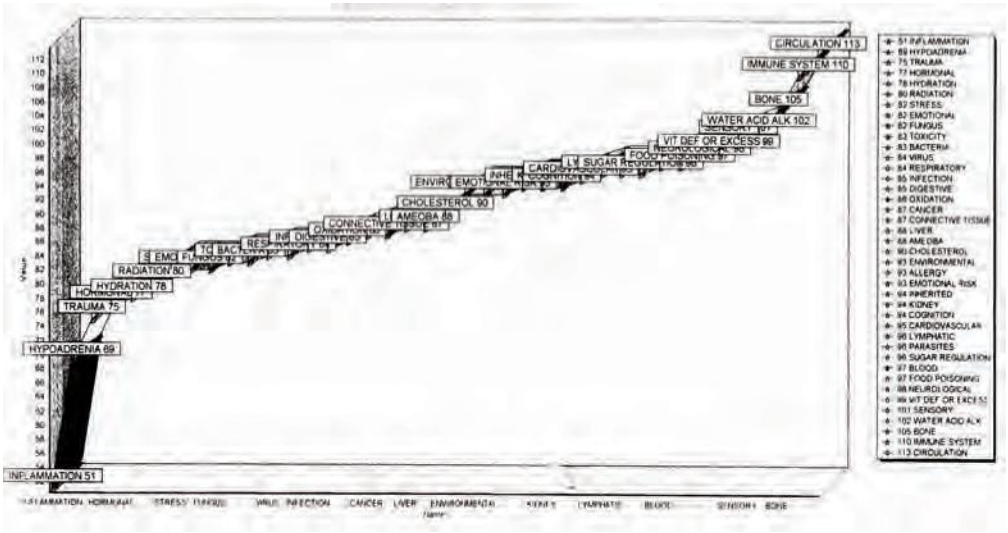


**Fig. 3. Risk of Disease Factors – E.D.**

Egor, a German Shepherd, age five, came with a benign prostatic hyperplasia. His risk factors' chart showed, in the superior extremity: radiation and water pH unbalance and in his inferior extremity: trauma, hypoadrenia, parasites. So we can say that his body was extremely stressed by radiation and the pH unbalance, but did not react properly to parasites and his trauma tolerance was very low.

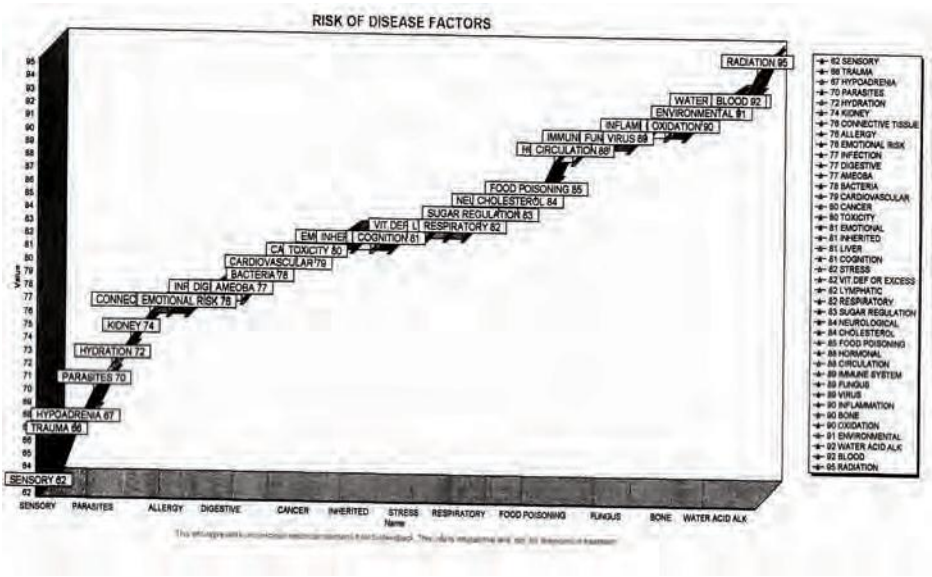


**Fig. 4. Risk of Disease Factors – Egor**

Milo, a Border Collie, age six, had bacterian prostatitis. The risk factors' chart offered in its inferior extremity: inflammation, infection and trauma. In the superior extremity we found: incomplete oxidation and water pH unbalance. This means his body had no reaction to infection and inflamation, but still responded to the aggresion of pH and incomplete oxidations.

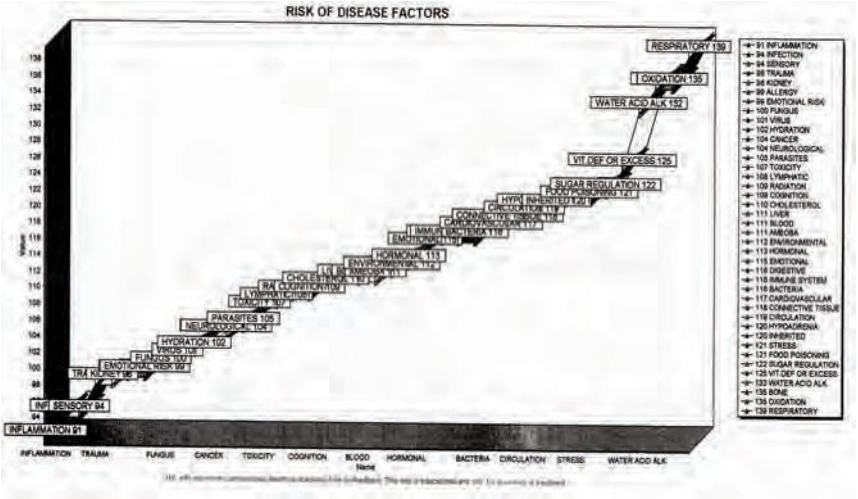


**Fig. 5. Risk of Disease Factors – Milo**

Sam, a Boxer, age four years and a half, had prostate cancer. We could determine on the superior extremity of the risk factors' chart: food

poisoning, meaning that the quality of the food was weak and incapacity of sugar regulation. In the inferior extremity of the chart could be seen: inflammation, radiation, infection, fungus and cancer. As we could see there, the body couldn't process the food and regulate his blood sugar, while his tissues were exposed to inflammations, infections, radiation and fungus aggresion and, of course, malignant degeneration.

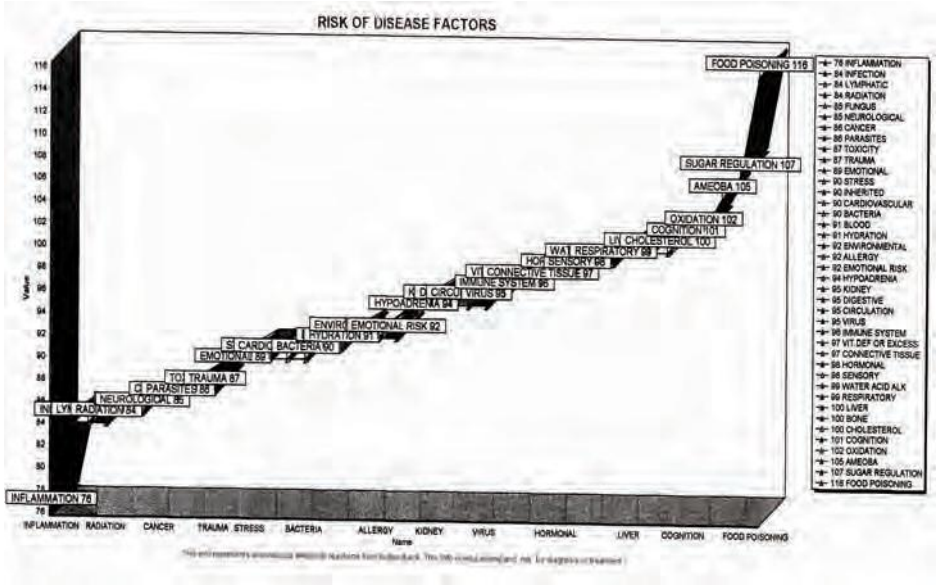


**Fig. 6. Risk of Disease Factors – Sam**

The presence of predisposition and weakneses for various environmental risk factors and tissue alterations gave the disease an easy ground.

## 3. CONCLUSIONS

3.1. There are no differences between the two species regarding the most common found risk factors in prostate disease.

3.2. There is a link between the place in the chart where we find the risks of interest and the seriousness of the disease. The more risks we find in the inferior extremity of the chart, the more serious is the disease or the outcome.

3.3. We could use the risk factors' chart to prevent the occurance and aggravation of prostate disease, knowing which risks we want to avoid or how the body reacts to them.

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# DYNAMICS OF BIOCHEMICAL BIOMARKERS FOR TESTICULAR FUNCTION CONSECUTIVE RATS EXPOSURE TO POTASSIUM DICHROMATE (THREE GENERATION EXPERIMENT)

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**Key words**: male rats, chromium VI, sex, hormones

## SUMMARY

The aim of the study was the evaluation of potassium dichromate impact on integrity and performances biomarkers of male reproductive system. The concrete objective was the estimation of potassium dichromate impact on biochemical biomarkers for testicular function, seric testosterone and LH level (three generation experiment).
The study was carried out for each generation on 28 white Wistar male rats mated with white Wistar female rats – ratio 2 females: one male, both exposed for three months before mating to potassium dichromate via drinking water as follows: $E_1$: 25 ppm Cr VI (LOAEL); $E_2$: 50 ppm Cr VI; $E_3$: 75 ppm Cr VI. The male rats from $F_1$ and $F_2$ generation were exposed *in utero*, via milk until weaning and via drinking water until sexual maturity at the same hexavalent chromium level.
The study pointed out: significant decrease of seric testosterone level comparative to control group and in inverse correlation to exposure level, in $F_0$, $F_1$ and $F_2$ generations; significant increase of seric LH level comparative to control group and in direct, correlation to exposure level, in $F_0$, $F_1$ and $F_2$ generations; decrease of seric testosterone level in $F_1$ generation comparative to $F_0$ generation, significant only in $E_1$ and $E_3$ groups; decrease of testosterone seric level in $F_2$ generation comparative to $F_1$ generation, significant only in E"$_2$ group; decrease of testosterone seric level in $F_2$ generation comparative to $F_0$ generation, significant only in E"1 and E"3 groups; significant increase of LH seric level in $F_1$ generation comparative to $F_0$ generation; increase of LH seric level in $F_2$ generation comparative to $F_1$ generation, significant only in E"$_3$ group; significant increase of LH seric level in $F_2$ generation, comparative to $F_0$ generation. In all experimental groups testosterone level was under physiological limits, and LH levels were over physiological limits.

Chromium is present in the environment in rocks, soils, animals, plants and in volcanic dust and gases. This metal exists in a series of oxidation states, but the most common and stable forms are elemental chromium, trivalent and hexavalent chromium. The chromium effects on health status are related to its valence state at the time of exposure. Trivalent and hexavalent compounds are thought to be the most biologically significant (ATSDR).

Trivalent chromium is an essential dietary mineral in low doses. Hexavalent chromium compound are men made, and his carcinogenicity has been demonstrated. Stainless steel production, welding,

electroplating, leather tanning, production of dyes, pigments and wood preservatives are the main industries responsible for hexavalent chromium compound production. Chromium VI is considered 1000 times more toxic to living beings than trivalent form (ATSDR).

There is growing concern on possible harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. This concern for endocrine disrupting chemicals is directed to both wildlife and humans. Several expert working groups have concluded that there is increasing evidence of adverse effects in human and wildlife reproductive health, and have discussed the hypothesis that chemicals in the environment have caused these endocrine mediated adverse effects (CSTEE).

The aim of this study was the evaluation of potassium dichromate impact on biochemical biomarkers for testicular function (seric testosterone and LH level) (three generations study).

## 1. MATERIALS AND METHODS

The study was carried out on three generations male rats. Rats were purchased from from Faculty of Medicine and Pharmacy *Biobase* Cluj Napoca.

$F_0$ generation was represented by 28 White Wistar male rats divided in three experimental and one control group. Male rats were exposed to potassium dichromate for three months before mating as followed: $E_1$: 25 ppm Cr VI (LOAEL) (EPA); $E_2$: 50 ppm Cr VI (2 x LOAEL); $E_3$: 75 ppm Cr VI (3 x LOAEL); control group received tap water without chromium content.

After mating with female rats exposed to potassium dichromate for the same period of time and hexavalent chromium level, blood samples were collected from male rats ($F_0$) for biochemical biomarkers determination.

Female rats continued to be exposed during gestation and lactation period to same levels of hexavalent chromium. After weaning, male pups ($F_1$ generation) were separated from female pups. Male pups continued to be exposed to same levels of hexavalent chromium trough drinking water for three more months (until sexual maturity). After this period of time seven rats from each group were mated with other females exposed for three months at same chromium levels. After mating blood sample were collected from male rats ($F_1$ generation) for biochemical biomarkers determination. Females continued to be exposed during gestation and lactation.

After weaning, male pups ($F_2$ generation) were separated from female pups. Males were exposed to the same levels of hexavalent chromium via placenta, milk and water until they reached sexual maturity, when blood samples were collected for biochemical biomarkers determination.

Blood samples for hormone assay were collected by cardiac puncture under anesthesia (Ketamine 50 mg/kg + Xylazine 5 mg/kg, intraperitoneal administration) from seven rats from each generation and groups. Plasma samples were separated by centrifugation, frozen and stored at -20$^o$ C until assessment. Seric testosterone and LH levels were determined by Tody Laboratories Bucharest (ISO 17025) using chemiluminiscence method. The amount of testosterone and LH was expressed as ng/ml.

All the animals have had free access to food and water. The study was performed in compliance with national and international law regarding animal welfare and ethics in animal experiments: 143/400/2002; 471/2002; 205/2004; 206/2004; 9/2008; 86/609/CEE.

The results were statistically analyzed by Anova method and Student test.

## 2. RESULTS AND DISCUSSIONS

The results are summarized in table 1 and figure 1.

*Table 1*

Seric testosterone and LH level (ng/ml) in $F_0$, $F_1$ and $F_2$ generation

| **Specification** / **Groups** | | **Testosterone** | | | | **LH** | | |
|---|---|---|---|---|---|---|---|---|
| | | **X±Sx** | **S.D.** | **CI 95%** | **Gr.** | **X±Sx** | **S.D.** | **CI 95%** |
| **Generation $F_0$** | C | 3.55±0.52 | 1.36 | 0.75 | C | 4.96±0.10 | 0.27 | 0.45 |
| | $E_1$ | 1.85±0.23$^*$ | 0.61 | 0.75 | $E_1$ | 5.88±0.14$^{**}$ | 0.36 | 0.45 |
| | $E_2$ | 1.12±0.44$^{**}$ | 1.17 | 0.75 | $E_2$ | 6.75±0.27$^{**}$ | 0.72 | 0.45 |
| | $E_3$ | 0.56±0.08$^{**}$ | 0.22 | 0.75 | $E_3$ | 7.07±0.29$^{**}$ | 0.78 | 0.45 |
| **Generation $F_1$** | C’ | 3.10±0.01 | 0.01 | 0.01 | C’ | 4.73±0.08 | 0.21 | 0.08 |
| | $E'_1$ | 1.25±0.01$^{**}$ | 0.01 | 0.01 | $E'_1$ | 6.22±0.01$^{**}$ | 0.01 | 0.08 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $E'_2$ | 0.88±0.01** | 0.02 | 0.01 | $E'_2$ | 7.34±0.01** | 0.01 | 0.08 |
| | $E'_3$ | 0.39±0.01** | 0.01 | 0.01 | $E'_3$ | 7.69±0.01** | 0.01 | 0.08 |
| **Generation $F_2$** | C" | 3.12±0.09 | 0.23 | 0.12 | C" | 4.63±0.12 | 0.32 | 0.20 |
| | $E"_1$ | 1.16±0.04** | 0.12 | 0.12 | $E"_1$ | 6.42±0.11** | 0.29 | 0.20 |
| | $E"_2$ | 0.72±0.05** | 0.12 | 0.12 | $E"_2$ | 7.48±0.10** | 0.25 | 0.20 |
| | $E"_3$ | 0.34±0.05** | 0.13 | 0.12 | $E"_3$ | 7.92±0.05** | 0.14 | 0.20 |

E/C: ns – not significant
*: p 0.05
**: p 0.01

Seric testosterone level decreased significantly in all individuals, from $F_0$ generation, exposed to potassium dichromate, comparative to control, ($E_1$/C: -47.88%, $p<0.05$; $E_2$/C: -68.45%, $p<0.01$; $E_3$/C: -84.22%, p 0.001) and inversely correlated to exposure level ($E_2/E_1$: -39.45%, p 0.05; $E_3/E_2$: -50%, p 0.05), significantly ($p<0.05$) only when exposure level increased from 25 to 75 ppm Cr VI ($E_3/E_1$: -69.72%, $p<0.05$).

In E' groups from $F_1$ generation seric testosterone level realized a significant ($p<0.01$) decrease comparative to C' group ($E'_1$/C': -59.67%; $E'_2$/C': - 71.61%; $E'_3$/C': -87.42%). Hormone level dynamics was indirectly, significantly ($p<0.01$) correlated to exposure level ($E'_2/E'_1$: -29.6%, $E'_3/E'_2$: -55.69%, $E'_3/E'_1$: -68.8%).

Comparative to $F_0$ generation seric testosterone level in $F_1$ generation was lower, significantly ($p<0.05$) only in $E'_1$ and $E'_3$ groups ($E'_1/E_1$: -32.43%, p 0.05; $E'_2/E_2$: - 21.42%, p 0.05; $E'_3/E_3$: - 30.35%, p 0.05).

Testosterone level in E" groups from $F_2$ generation suffered a significant ($p<0.01$) reduction comparative to control group ($E"_1$/C": -62.83%; $E"_2$/C": -76.93%; $E"_3$/C": -89.11%). Testosterone dropping off was indirectly, significantly ($p<0.01$) correlated to exposure level ($E"_2/E"_1$: -37.94%, $E"_3/E"_2$: -52.78%, $E"_3/E"_1$: -70.69%).

Comparative to $F_1$ generation, testosterone level in E" groups from $F_2$ generation decreased, being significant (p 0.05) only in $E"_2$ group ($E"_1/E'_1$: -0.92%, p 0.05; $E"_2/E'_2$: -18.19%, p 0.05; $E"_3/E'_3$: -12.82%, p 0.05).

In exposed rats from $F_2$ generation testosterone level was lower comparative to $F_0$ generation but significant only in E"$_1$ and E"$_3$ groups (E"$_1$/E$_1$: -37.29%, p 0.01; E"$_2$/E$_2$: -35.71%, p 0.05; E"$_3$/E$_3$: -39.28%, p 0.05).

In $F_0$ generation all exposed groups presented significant increase ($p<0.0001$) of seric LH concentration comparative to control one (E$_1$/C: +18.54%; E$_2$/C: +36.08%; E$_3$/C: +42.54%), directly and significantly correlated to exposure level, excepting, not significantly ($p>0.05$), when exposure level increased from 50 to 75 ppm Cr VI (E$_2$/E$_1$: +14.79%, $p<0.05$; E$_3$/E$_2$: +4.74%, $p>0.05$; E$_3$/E$_1$: +20.23%, $p<0.01$).

In $F_1$ generation all experimental groups (E') had significantly ($p<0.01$) higher seric LH level comparative to control group (C') (E'$_1$/C': +31.50%; E'$_2$/C': +55.17%; E'$_3$/C': +62.57%). LH level in exposed groups was directly and significantly ($p<0.01$) correlated with exposure level (E'$_2$/E'$_1$: +18%, E'$_3$/E'$_2$: +4.76%, E'$_3$/E'$_1$: +23.63%).

Comparative to males from $F_0$ generation, LH seric level was significantly ($p<0.05$) higher in $F_1$ generation (E'$_1$/E$_1$: +5.78%; E'$_2$/E$_2$: +8.74%; E'$_3$/E$_3$: +8.76%).

LH level in $F_2$ generation increased significantly (p 0.01) in E" groups comparative to control group (E"$_1$/C": +38.66%; E"$_2$/C": +61.55%; E"$_3$/C": +71.05%). Correlation between hormone level rising and exposure level was direct and significant (p 0.01) (E"$_2$/E"$_1$: +16.51%, E"$_3$/E"$_2$: +5.88%, E"$_3$/E"$_1$: +23.36%).

The two generation comparison reveals that LH seric level in $F_2$ generation was higher in exposed groups comparative $F_1$ generation, significant (p 0.01) only in E"$_3$ group (E"$_1$/E'$_1$: +3.21%, p 0.05; E"$_2$/E'$_2$: +1.90%, p 0.05; E"$_3$/E'$_3$: +2.99%, p 0.01).

In $F_2$ generation seric LH level increased significantly comparative to $F_0$ generation (E"$_1$/E$_1$: +9.18%, p 0.01; E"$_2$/E$_2$: +10.81%, p 0.05; E"$_3$/E$_3$: +12.02%, p 0.01).

Comparative to physiological limit (2-3 ng/ml – Krinke, 2000) testosterone seric level was lower than maximum limit in E, E' and E" groups (E$_1$/Ph: -26%; E$_2$/Ph: -55.2%; E$_3$/Ph: -77.6%; E'$_1$/Ph: -50%; E'$_2$/Ph: -64.8%; E'$_3$/Ph: -84.4%; E"$_1$/Ph: -53.6%; E"$_2$/Ph: -71.2%; E"$_3$/Ph: -86.4%).

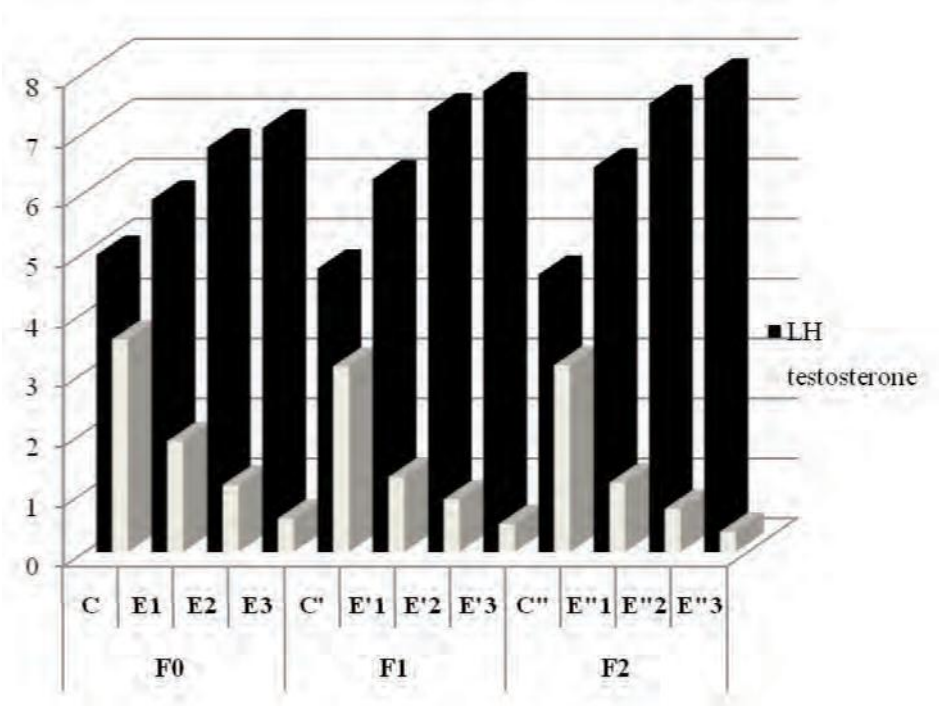


**Fig. 1. Sex hormone levels dynamics in $F_0$, $F_1$ and $F_2$ generations**

In all generations LH seric level was higher than the physiological limit (Ph) (0.5 ng/ml – Krinke, 2000) in all groups but more evident in E, E' and E" groups ($E_1$/Ph: +1076%; $E_2$/Ph: +1250%; $E_3$/Ph: +1314%; $E'_1$/Ph: +1144%; $E'_2$/Ph: +1368%; $E'_3$/Ph: +1438%; $E''_1$/Ph: +1184%; $E''_2$/Ph: +1396%; $E''_3$/Ph: +1484%).

Results regarding testosterone level were in accordance with Ernst and Bonde, Yousef *et al*. and Chandra *et al*. results (Ernst and Bonde, 1992; Yousef *et al*., 2006; Chandra *et al.,* 2007).

Similar results concerning LH seric level were reported by: Ernst and Bonde, Li *et al*. and were controversial to Chandra *et al*. results (Ernst and Bonde, 1992; Li *et al*., 1999; Chandra *et al*., 2007).

The major site of testosterone synthesis is represented by Leydig cells. Decrease of testosterone level can be associated with Leydig cell alterations, described by some authors (Aruldhas *et al.,* 2005; Muselin *et al.,* 2007; Pereira *et al*., 2002) and by research team too (Rankov *et al*., 2009 and Trif *et al.,* 2009).

Testicular lipid peroxidation increase, induced by Cr VI treatment, is also involved in low seric testosterone concentration, as immoderate generation of reactive oxygen species is associated with Leydig cell impairment (Chandra *et al*., 2010).

LH level is regulated by negative feed-back by testosterone. That explains the increasing dynamics of LH levels.

Also, the increase of seric LH emphasized that pituitary gland is still functional, though there are experiments that pointed out the negative impact of Cr VI on pituitary gland structure and functionality, respectively on hormones secretion (Quinteros *et al*., 2007).

## 3. CONCLUSIONS

The exposure of adult male rats to potassium dichromate (Cr VI) in drinking water (25, 50 and 75 ppm Cr) along three generations determined:

1.1. Significant decrease of seric testosterone level comparative to control group and in inverse correlation to exposure level, in $F_0$, $F_1$ and $F_2$ generations;

1.2. Significant increase of seric LH level comparative to control group and in direct, correlation to exposure level, in $F_0$, $F_1$ and $F_2$ generations;

1.3. Decrease of testosterone seric level in $F_1$ generation comparative to $F_0$ generation, significant only at 25 and 75 ppm Cr VI exposure level;

1.4. Decrease of testosterone seric level in $F_2$ generation comparative to $F_1$ generation, significant only at 50 ppm Cr VI exposure level;

1.5. Decrease of testosterone seric level in $F_2$ generation comparative to $F_0$ generation, significant only 25 and 75 ppm Cr VI exposure level;

1.6. Significant increase of LH seric level in $F_1$ generation comparative to $F_0$ generation;

1.7. Significant increase of LH seric level in $F_2$ generation comparative to $F_1$ generation, significant only at 75 ppm Cr VI exposure level;

1.8. Significant increase of LH seric level in $F_2$ generation, comparative to $F_0$ generation.

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# RESEARCH CONCERNING THE INFLUENCE OF THE STUNNING SYSTEM ON MEAT QUALITY

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**KEYWORDS**: stunning, lamb meat, quality, assessment, comparison.

**SUMMARY**

The main objective of this study was to establish the effect of different types of stunning methods on the quality of refrigerated meat obtained from lambs, at 24 h and a week post-mortem. The main stunning methods included in the study were: electrical stunning (ES), $CO_2$ stunning and slaughter without stunning. The measurements for quality of the obtained meat included: pH, water holding capacity (WHC), cooking losses (CL), shear force (SF) and drip loss (DL). No significant differences were observed in any of the established parameters for evaluation at 24 h, but at a week post-mortem, the meat quality showed high differences, thus being evidently affected, considering each stunning technique: pH, CL and DL were lower considering slaughter without stunning and stunning with $CO_2$. SF was affected in the case of meat obtained by ES.

Lamb meat is considered a "luxury food", being largely consumed in the Mediterranean countries, where sheep meat production and rearing of this species is concentrated [5,10]. Although the price is considered the main factor when deciding to buy such a product, the meat characteristics seem to be more important, and also the food safety and the final quality [6,9]. Considering the stunning methods, the welfare of the animals is another important topic. Stress previous slaughter is considered of great interest, due to its influence on the image of the product, as well as its effect on meat quality [2]. According to European law (EU Council Directive 93/119/EC) electrical stunning in sheep is a compulsory procedure to ensure an adequate state of unconsciousness in animals previous to slaughter. However, some disadvantages have been described, in relation to this stunning system, e.g., haemorrhage, blood splash, bone fractures, lowered safety for slaughter-men etc. [10]. Other methods, such as the one using $CO_2$ was discovered to contribute to a better preservation of the food product, improving the bleeding in the carcass, as a consequence of the increase in the heart rate frequency and blood pressure [12, 13]. The present study had the main objective to assess the effect of the stunning method on the meat quality of lamb at 24 h (initial meat quality) and at a week (7 days), the maximum time permitted for sale.

## 1. MATERIALS AND METHODS

The research protocol was performed in agreement with Executive Committee 86/609/CEE, concerning the protection of animals used in research and for scientific purposes. Lambs were transported form farm to the abbatoir, and held in pens for 15 h without feed but access to water. Animals were distributed into three groups, considering the selected methods of slaughter: ES, $CO_2$ and slaughter without stunning. ES was performed at 110 V, 5 s. $CO_2$ stunning was performed using gas in groups of five in the box, 90% $CO_2$ at the bottom of the well. After slaughter, carcasses were chilled at 4$^{o}$C for 24 h.

The measurements were performed on *Longissimus dorsi* muscle, which was removed at 24 h post-mortem, from the carcass and divided in two pieces, from T7 to T11. One of them was used to evaluate the initial meat quality and the second to assess it at a week difference, packed in a plastic tray and kept at 2$^{o}$C. Before removing *Longissimus dorsi* muscle, pH was measured on the carcass.

Water holding capacity was expressed as percentage of free water, CL as the percentage of loss related to the initial weight, followed by SF, analyzed using a texture analyzer. For the latter, each meat piece was place in a polyethylene bag in a water bath, at 75$^{o}$C for 30 minutes. After drying, they were subjected to cut, with the texture analyzer, and all data was recorded. At a difference of a week, the same parameters were analyzed, for the duplicate samples.

## 2. RESULTS AND DISCUSSIONS

The values for pH (table 1) were the lowest for $CO_2$ stunning, due to residual gas in the muscle. At 7 days post-mortem, a higher pH value was observed in the ES and $CO_2$ stunning, than in unstunned lambs. This may be possible due to a potential high level of catecholamines, that reduce the lactic acid production post-mortem, due to an increase of glycogenolysis.

Considering WHC (table 2 and fig. 2), at 24 h post-mortem, there were no significant differences, but after 7 days, a decrease was observed on CO2 stunning group. This may be due to the release of water and juiciness. An increase in adrenaline in the bloodstream of animals due to stress or by external injection has an important effect on muscular proteolysis since it decreases myofibrillar space and therefore increases water loss (minor WHC). On the other hand, cooking loss (CL) for the gas-stunned lambs was intermediate between electrical and un-

stunned animals. The ES group caused the highest CL, showing significant differences between 24 h and 7 days. Drip loss (DL) was higher in stunned animals than in the un-stunned group. Perhaps, in stunned animals, ageing of meat starts earlier and also proteolysis of muscle implies shorter sarcomeres associated with an increase in DL as a consequence of the stunning system. On the other hand a decrease in the glycogen level (and therefore a high pH) just before slaughter supposes a higher value of drip loss like in the stunned lamb groups (ES and $CO_2$ stunning), which showed a high pH than the group of unstunned animals. As regards shear force (SF), stress previous to slaughter has no effect on tenderness values. According to our results, no significant differences were found among the three groups of TS.

***Table 1***

***Effect of type of stunning on the pH (mean values) of meat***

| Parameter | Time post-mortem | ES | $CO_2$ stunning | No stunning |
|---|---|---|---|---|
| pH | After dressing | 6,69 | 6,42 | 6,74 |
| | At 24 h | 5,45 | 5,61 | 5,39 |
| | At 7 days | 5,58 | 5,54 | 5,33 |

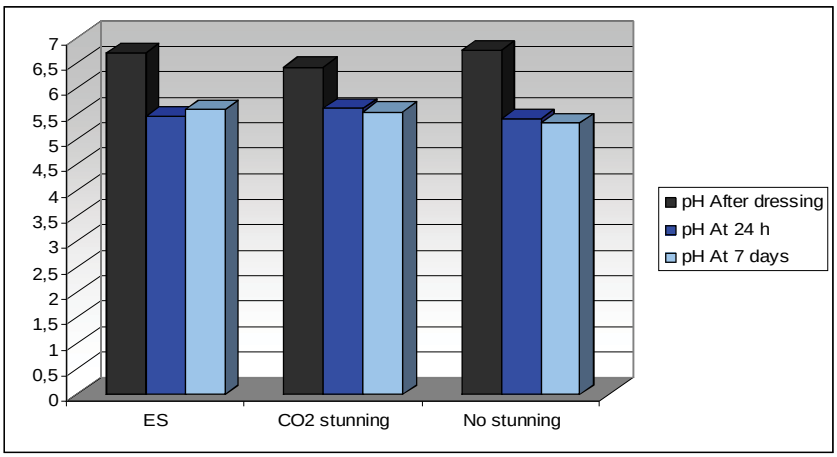


**Fig. 2 - Effect of type of stunning on the pH (mean values) of meat**

**Table 2 –**
***Effect of type of stunning method on water holding capacity, cooking loss, drip loss and shear force of meat obtained from lamb***

| Parameter | Time post-mortem | ES | $CO_2$ stunning | No stunning |
|---|---|---|---|---|
| **WHC (% water expelled)** | 24 h | 15,12 | 15,82 | 16,74 |
| | 7 days | 17,58 | 27,13 | 18,10 |
| **CL (%)** | 72 h | 12,09 | 7,54 | 9,68 |
| | 7 days | 15,72 | 11,84 | 6,15 |
| **DL (%)** | 7 days | 1,55 | 1,87 | 0,69 |
| **SF (N/cm$^2$)** | 72 h | 77,85 | 74,38 | 84,19 |
| | 7 days | 45,17 | 53,82 | 74,57 |

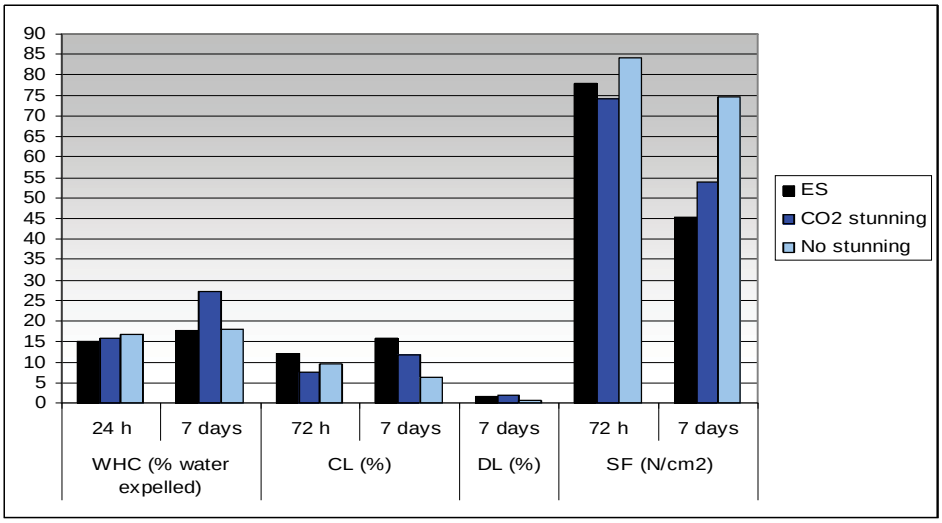


**Fig. 2 - Effect of type of stunning method on water holding capacity, cooking loss, drip loss and shear force of meat obtained from lamb**

## 3. CONCLUSIONS

The stunning method effect on meat quality of lambs had been determined in this study, in order to clarify if the final quality of this product may be affected in a way or another, due to stress or other status of the animals. In general, pH values showed no effect of the stunning method. At 7 days post-mortem, electrical and $CO_2$ stunning produced higher CL and DL than those slaughtered without previous stunning. This fact shows that the stunning method could actually accelerate the

ageing of meat and thus favoring changes in some quality parameters, such as water losses.

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# RESEARCH CONCERNING THE QUALITATIVE AND QUANTITATIVE DETECTION OF MEAT ADULTERATION BY USING A PCR ASSAY

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**KEYWORDS**: poultry, pork, meat, PCR, authenticity

**SUMMARY**

A classic PCR technique was used in order to establish the authenticity of poultry and pork meat in different binary mixtures of minced meat purchased from local stores. The concentration of pork in poultry meat, in samples of 100 g each, was different: 0,5%, 1%, 2%, 2,5%, 5%, 10%, 25%, 50%, 75%, 90%, 95 %, 99% and 99,9% (w/w). After the DNA extraction, the next step was the amplification of the DNA sequences, and for the analysis of PCR products (fragments), we used agarose gel electrophoresis. The results showed the specific detection and quantification for each type of meat type by origin, in comparison to validation samples (100 % pork and 100 % poultry). The specificity of the technique is high, thus being declared a very useful tool for future authenticity tests.

In order to avoid unfair competition among producers and allowing consumers to have accurate information about the acquired products, food composition and and authenticity assessment is a very important issue at the present moment [2,8,9]. Following the European Union labelling regulations, meat products should be accurately labelled regarding their species content. Quality and authenticity evaluation in meat products encompasses many issues, such as the fraudulent substitution of higher commercial value meats by lower value meats [5,4]. A series of analytical methods were elaborated and suggested for the identification of meat species in mixed samples, including different protein-based methods such as high-performance liquid chromatography, electrophoretic techniques and enzyme-linked immunosorbent assays [3]. The species of origin in raw meat can be identified by using most of these protein-based methods, some authors referred that they are significantly less sensitive in the evaluation of thermally processed foods because of specific epitopes alterations [1,7,10]. DNA molecules have been chosen as target compounds due to their high stability compared to proteins, and to their ubiquity in every type of cell. The analysis of DNA coupled with polymerase chain reaction (PCR) presents a fast, sensitive and highly specific alternative to protein-based methods [2,6,15]. PCR technique uses a fluorescent

dye, able to give a proportional results to the initial amount of DNA chosen as target, facilitated by the real-time monitoring of amplification products for each cycle. The objective of this present work was to assess the possibility to use PCR technique as a tool for establishing in a precise manner the authenticity of processed food products, containing poultry and pork meat.

## 1. MATERIALS AND METHODS

The samples for the analysis consisted of poultry and pork meat pieces. After purchasing the meat samples from a local store, they were cut and minced separately. Afterwards, there were obtained a series of binary mixtures, used as reference samples, containing 0,5%, 1%, 2%, 2,5%, 5%, 10%, 25%, 50%, 75%, 90%, 95 %, 99% and 99,9% (w/w) of pork in poultry meat were prepared to a final weight of 100 g. The next step was the addition of sterile phosphate-buffered saline in a quantity of 10 ml, and each mixture was homogenized using a blender. In order to avoid contamination, each mixture was processed separately. For validating the estimation of the analysis, a new series of samples was prepared, with 1%, 2,5 %, 5%, 10%, 20% and 40% of pork in poultry meat (w/w). All the samples were kept at -18$^{o}$C…-20$^{o}$C until DNA extraction.

A quantity of 150 mg of the samples grounded and homogenized was transferred in a reaction tube of 2 ml, and TNE extraction buffer was added in a quantity of 750 µl, 150 µl of 5 M guanidine hydrochloride solution and 100 µl proteinase K. An incubation step was realized at 55$^{o}$C, for 4 h, with occasional stir, and centrifugation followed, for 15 minutes at 12,000 rpm. A quantity of 450 µl of the supernatant was mixed with 1,5 ml of DNA purification resin. The mixture was eluted and the resin was washed with 10 ml of isopropanol solution (50 %, v/v). After the elution column dried, the DNA was eluted by centrifugation, (3 min, 10,000 rpm), with 150 µl Tris-EDTA buffer, at 60$^{o}$C into another reaction tube. In order to evaluate the quality of the DNA, electrophoresis was used, in agarose gel 2,5 %, in TAE buffer, for 60 minutes, stained with ethidium bromide and destained in distilled water for 30 minutes. The visualization was performed in UV light.

DNA was quantified by spectrophotometry, the concentration being determined by UV absorbance at 260 nm. The PCR amplification step was carried out in 25µl total reaction volume, with 2 µl of DNA extract. For the pork meat samples, the composition was: 25 ng DNA, 25 mM

Tris-HCl, 70mM KCl, and 0,5 μM of each primer. For poultry meat samples, reaction mixture was: 75 ng DNA, 25 mM Tris-HCl, 70 mM KCl, and 0,75 μM of each primer. The PCR amplification was performed in a thermal cycler, Roche 2.0. The programs used were the following: initial denaturation at 94°C, for 5 minutes; 35 and 38 cycles for pork and poultry meat samples, at 94°C for 30 s, 60°C for 60s and 72°C for 60 s; and the final extention was at 72°C for 5 minutes. The fragments were afterwards analyzed by electrophoresis in a 2,0% agarose gel, for 60 minutes at 120 V, stained with ethidium bromide, and destained with distilled water, for 30 minutes.

## 2. RESULTS AND DISCUSSIONS

The quality of extracted DNA was assessed by gel electrophoresis, in order to verify the integrity of the DNA, and what are the parameters that depend on the evaluated material, the processing status and the method used for DNA extraction. The results showed various smears of short and long DNA, leading to the conclusion that the DNA was not degraded in such a major degree. The purity of the DNA was high, with a yield of 716-1034 ng/μl. The amplification of the DNA extracts for both meat species led to PCR fragments of 149 (pork) and 183 bp (poultry) (Fig.1 and 2). Also, it was verified the sensitivity of the technique, using all the samples defined by various concentrations, until 0,5%.

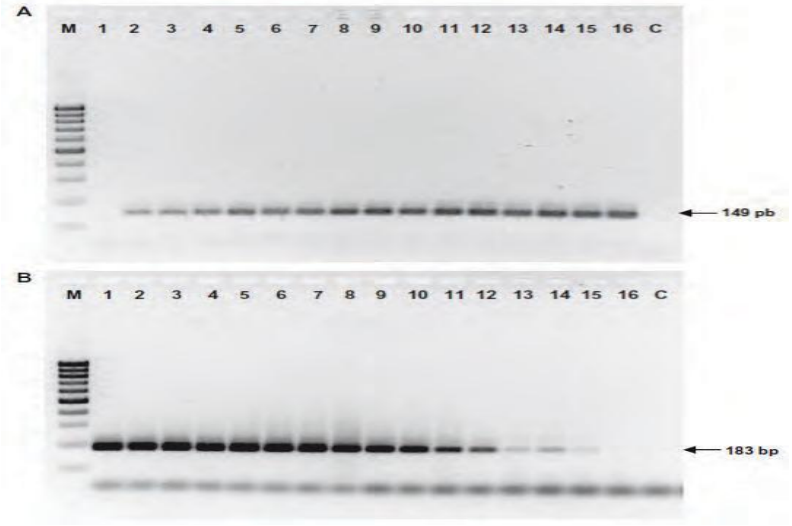


***Fig.1. The results of agarose gel electrophoresis for the PCR products of pork (I) and poultry (II); 1 – 100% poultry; 2-15: 0,5%, 1%, 2%, 2,5%, 5%, 10%, 25%, 50%, 75%, 90%, 95 %, 99% and 99,9% (w/w) for pork addition; 16 – 100% pork, and C: negative control.***

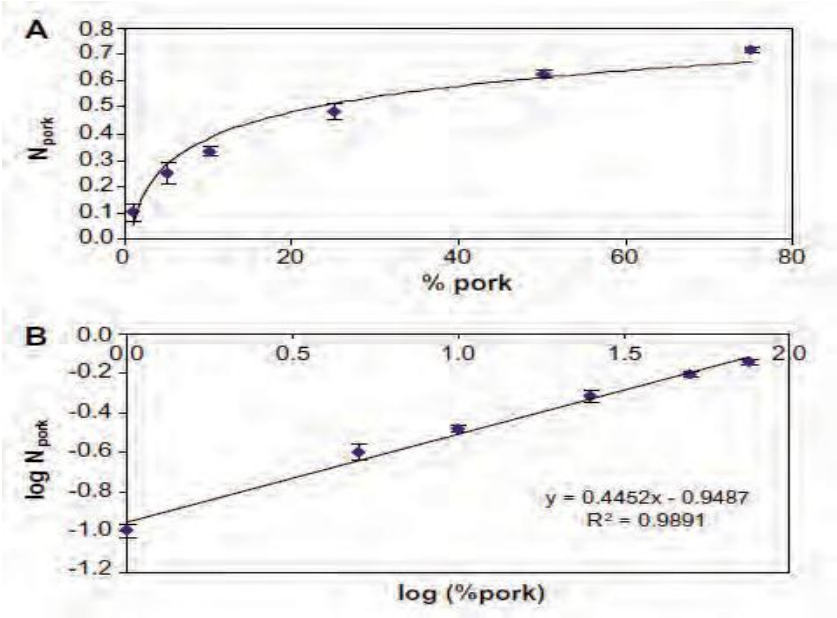


***Fig. 2. Normalised calibration curves for the estimation of poultry meat adulteration with pork.***

## 3. CONCLUSIONS

3.1. The necessity of clear and precise methods for establishing the authenticity of food products, especially those of animal origin had become a very important point for research.

3.2. The PCR technique provides a very useful technique to use in this particular case, even though the costs are high. By amplification and electrophoresis with agarose gel, the meat products authenticity for the samples analyzed during this study was determined in a qualitative, but more important, a quantitative way.

3.3. The applicability of this technique was demonstrated by using binary mixtures between poultry and pork minced meat. The results showed that PCR technique is the answer for the necessity of a method to establish the authenticity of meat products.

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# STUDY REGARDIND THE PREVALENCE OF ENDOPARASITARY INFESTATION OF DONKEYS FROM A SHELTER

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**Key words**: donkeys, fecal sample,

**SUMMARY**

Donkeys are still used, in some areas, especially for transportation of goods. Their number is smaller compared with horses, and studies regarding parasitic infestation of donkeys in our country are rare. The present study has followed the evaluation of infestation prevalence in donkeys in a shelter from the county of Constanta, from 2009 to 2010. 92 samples of fecal matters have been examined, from which 68.48% have been positive and 31.52% negative. The fecal samples exam has indicated a 96.83% prevalence of *Strongyle spp.* infestation, 6.35% *Parascaris spp.* and 6.35% *Eimeria spp*.

Donkeys (*Equus asinus* Linnaeus) were wild animals until their domestication by the Egyptians, being used for a variety of work. In our days, they still represent the main work animals in different regions of the world, especially in Africa, South Asia and Latin American (Colunga et al, 2005; Kuzmina and Kuzmin, 200; Hosseini et al., 2009). It is considered that, on a global level, approximately 44 million donkeys are used mainly as work animals (Kuzmina and Kuzmin, 2008). In our country, their number is small compared to horses, being used by shepherds for watching the sheep herds and less for carrying goods, especially on mountain regions.

Parasites are found everywhere in the world, especially in areas with abundant precipitations, donkeys representing a fine host for numerous species of parasites, becoming an infestation reservoir for the nearby horses. Generally in equines, endoparasitism represents a medical problem, nematodes being known for their high prevalence compared to cestodes, trematodes and sporozooses (Uslu et al., 2007; Umur and Açici, 2009). Of the nematodes, strongyles represent one of the main endoparasitosis, evolving as respiratory (*Dictyocaulus arnfieldi*) or digestive (*Strongylus spp.*) disease. Lungworms cause respiratory problems due to their migration through the host body, but in donkeys they usually evolve asymptomatic although they represent the species where the adult stage of the nematode takes place. The infested animals will release eggs with embryos, causing environmental contamination

(Mitrea, 2002). Intestinal strongyles cause digestive problems and in severe infestation cause emaciation, debility and even death (Pilarczyk et al., 2010). The ascarids parasite in the small intestine causing diarrhoea, colic due to intestinal obstructions. In youth it can cause severe respiratory problems due to larvae migration on entero-pneumo-tracheo-intestinal levels. Pinworms cause discomfort due to adult females that deposit eggs perianal causing itching and anal lesions caused by rubbing against objects (Niculescu and Didă, 1998). Tapeworms parasite in the small intestine, causing irritations and blockage of the ileo-caecal valve and colic.

In our country, studies regarding parasitic infestation in donkeys are rare, therefore the study shown here proposes to evaluate endoparasitic infestation in donkeys from a shelter.

## 1. MATERIAL AND METHODS

Between 2009 and 2010, inside the Laboratory of Parasitology of the Faculty of Veterinary Medicine Bucharest, a number of 92 samples of feces were collected from donkeys within a shelter in Constanta County. The fresh samples of feces were collected individually, wrapped in plastic bags and transported to the laboratory for parasitic examination, were they were kept in the refrigerator (4° C) until examination. The samples were put under microscope for quality evaluation by the standard procedure through the flotation method, the eggs being identified on the morphology described in scientific literature. The McMaster method was used to determine the egg/gram load.

## 2. RESULTS AND DISCUSSION

The coprological examination carried on a number of 92 fecal samples has revealed a 68.48% prevalence of parasitic infestation. Among parasites determined in donkeys, prevalence of *Strongylus sp., Parascaris sp.* şi *Eimeria sp.* was 96,83%, 6,35% and 6,35%, respectively. After the quantity examination, it has been noticed that 60.32% of pozitive animals showed a light infestation, 17.46% presented medium infestation, 14.29% with high infestation and 7.94 % with severe infestation (fig.1).

Studies regarding endoparasitic infestation in donkeys were carried in a large number of countries, especially where they are met in large numbers, and using them as work animals is often. Therefore, Gül et al. (2003), during a study regardind tapeworm in equines from different

parts in Turkey, have obtained a 77.3% prevalence of parasitic infestation in donkeys. Another study, Umur et al.(2009) have identified a 96.77% prevalence of parasitic infestation in donkeys from Central Back Sea region. Uslu and Guçlu (2007) have indentified a 100 % prevalence of infestation in donkeys from Konya, Turkey. In Greece, Sotiraki et al (1997), have examined 150 donkeys and identified a 75.7% prevalence of parasitic infestation. In Ukraine, Kuzmina and Kuzmin (2008), have studied the component of the species of strongyles in donkeys and observed 100 % prevalence of parasitic infestation. In Etyopia, Ayeli et al. (2006) have obtainded an 100% prevalence of parasitic infestation within the examined donkeys, and Getachew et al. (2010) have obtained 99% prevalence. In India, Shrikhande et al. (2009) have examined 82 donkeys from Nagpur region and noticed a prevalence of 82.9% of endoparasitic infestation. Burder et al. (2010), in Mexico, examined a feces from a number of 177 donkeys and finding 80% of them to be pozitive.

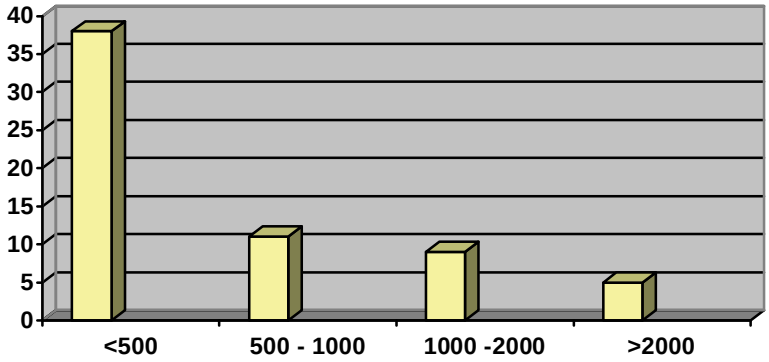


**fig 1. Fecal load of eggs from positive animals (epg)**

As shown in the previous data, the values recorded in thist study are smaller compared to those obtained in other countries.This can happen due to geographical, climate and animal care differences, specific to each area.

Regarding the quantity examination, made by establishing the epg of fecal samples, it has been observed that the majority of animals showed mild infestation of under 500 eggs per gram. Acording to Soulsby (1982) quoted by Getachew et al. (2010), alod up to 500 eggs per gram means mild infestation, between 500 and 1000 eggs per gram means moderate infestation, between 1000 and 2000 eggs means high infestation and over 2000 eggs per gram means severe infestation. Our results are

similar to those obtained by Kuzmina and Kuzmin (2008), who found mild infestation with values between 50 and 250 epg. Burder et al. (2010) and du Toit et al. (2008), in Mexico, have noticed a moderate load of parasitic infestation and Getachew et al. (2010), in Etyopia, obtained a high load within the majority of the examined animals. The differences in the intesity of infestation could be determined by microclimate conditions in which the animals are kept, feeding practices and antihelmintic treatments applied.

The animals in the study shown here were kept in a shelter and received antiparasitic treatments when introduced in the population, which explains the values obtained.

## 3. CONCLUSIONS

3.1. The study shown here has revealed the presence of a number of three species of intestinal parasites in donkeys from the mentioned shelter, revealing nonetheless the fact that the majority of animals, 60.32%, showed mild infestation.

3.2. The checking for intestinal endoparasites is an important aspect in maintaining animal healthcare, therefore it is recommended to check the newly arrived animals and treating the ones who were positive to prevent the spread of the infestation to healthy animals.

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# STUDY ON NATURAL INFESTATION WITH HARD TICKS ON DOGS IN BUCHAREST

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**Key words:** hard ticks, dogs, natural infestation

**SUMMARY**

Ticks from the *Ixodidae* family are arthropode parasites that have their body covered with a relatively hard shell, from which the name "hard ticks". They represent the most important vector group from Phylum *Arthropoda*, being responsible for the maintenance and transmission of pathogen agents to humans and animals, including a large number of protozoa, bacteria and viruses species. Lately, a significant growth of canine babesiosis has been observed in the southern part of the country, the disease being transmitted from ticks of the *Ixodidae* family. In the following paper, types of ticks collected from the body of dogs with babesia disease are being presented. The study was carried in Faculty of Veterinary Medicine Bucharest Clinic from September 2009 to August 2010, on a number of 83 dogs from which 418 ticks have been collected. Following the examination of the collected ticks, it has been found that they belonged to the *Demacentor reticulatus* species - 67.22% (281/418) and *Rhipicephalus sanguineus* - 32.78% (137/418). The majority of ticks were females – 84.45% (353/418), from which 67.99% (240/353) belonged to *D. reticulatus* species and 32.01% (113/353) belonged to *Rh. sanguineus* species. The males represented 15.55% (65/418) of the total ticks collected, from which 63.08% (41/65) belonged to *D.* reticulates species and 36.92% (24/65) belonged to *Rh. sanguineus* species.

Ticks from the *Ixodidae* family are ectoparasitic arthropods found in both wild and domestic animals and in humans. The ticks are large in size with a shield on the dorsal side of the body. The dorsal shield covers the entire surface of the body in adult males and a part of the body in adult females. The *Ixodidae* ticks are temporary parasites, with long periods of free life and periods of parasitic life (Cosoroabă, 2005). In the parasitic period, the ticks feed with blood. Because they feed on the blood of the host they parasite, the ticks are responsible for the maintenance and transmission of pathogen agents, including several species of protozoa, bacteria and viruses (Jongejan and Uilenberg, 2004; Dantas-Torres F., 2010). One of the most common transmitted diseases is babesiosis, the ticks playing a major part in the epidemiology of this hemosporidiosis, being the definitive hosts. Dogs represent an important source of feeding for the *Ixodidae* ticks and a reservoir for *B. canis* (Shaw et al., 2001; Menn et al., 2010). The increased mobility of pets and the tick's ability of finding ways in the recent climate conditions have resulted in the quick spread of zoogeographical intervals for some of the ticks (Shaw et al., 2001). Lately, there has been a significant

growth of canine babesiosis cases in the southern part of the country, with variable clinic manifestations (Tudor et al., 2008). Taking this aspect into consideration, a study regarding the natural infestation with ticks in dogs from Bucharest and surrounding areas, has been considered necessary. In the following paper, types of ticks collected from the body of dogs with babesia disease are being presented.

## 1. MATERIAL AND METHOD

Between September 2009 and August 2010, inside the clinic of the Faculty of Veterinary Medicine Bucharest, a number of 418 ticks were collected from the body of 83 dogs that have been submitted to the doctor with clinical symptoms of babesiosis. The collected ticks were examined with a stereomicroscopic lens and identified using the standard procedures as described by Didă et al. (2000).

## 2. RESULTS AND DISCUSSION

After examining the collected ticks, two species were identified, *Dermacentor reticulatus* with a number of 281 individuals, representing 67.22% and *Rhipicephalus sanguineus* with 137 individuals, representing 32.78%. From the total number of 418 ticks, 353 (84.45%) were females and 65 (15.55%) were males. The majority of females belonged to *D. reticulates*, with a number of 240 individuals (67.99%; 240/353) and *Rh. sanguineus* with 113 individuals (32.01%; 113/353). From the total number of 65 males, 41 belonged to *D. reticulatus* representing 63.08 %, and 24 belonged to *Rh. sanguineus* representing 36.92%.

All the collected ticks were attached to the skin of the examined dogs, with the following distribution: 159 from the head region representing 38.04%, 99 from the limb region (axilar and inguinal included) - 23.68%, from the neck level - 20.81% and 73 from the thoracic and abdominal region - 17.46%. The number of ticks indentified in each dog varied from 1 to 14 individuals, and on one dog, that had been in a hunting recently, 21 individuals were found.

The ticks found in this study were *D. reticulatus* and *Rh. sanguineus*, the obtained results showing that the predominant was *D. reticulatus*.The bio-ecological characteristics of the two identified species are related to environmental conditions of the area where the examined animals came from, the southern part of the country (Mitrea, 2002).

Studies carried in different parts of the globe have shown that the existing species of ticks in dogs, as their highly varied prevalence by season or geographically, are influenced by abiotic factors (structure of the habitat, climate conditions) and biotic factors (host population) (Dantas-Torres, 2010; Gray et al., 2009). In Hungary, Földvári and Farkas (2005) identified 6 species of ticks from a total number of 900 individuals, collected from 310 dogs over a period of 2 years. Of those, *D. reticulatus* had the largest prevalence (48.09%), followed by *Ixodes ricinus* (43.02%), *I. canisuga* (5.6%), *Haemophysalis concinna* (2 %), and one individual belonging to *D. marginatus* and the other to *I. hexagonus*. In Polland, Zygner and Wedrychowichz (2006), within a study carried on 316 dogs, have identified two species of ticks represented by *D. reticulatus* (64.6%) and *I. ricinus* (35.4%). In Italy, Torina et al. (2006) collected 561 ticks from dogs, identifying 99.8% belonging to *Rhipicephalus* species (*Rh. sanguineus* – 50.4%, *Rh. turanicu* – 48.5% and *R. bursa* – 0.9%) and one individual belonging to *Ixodes hexagonus*. In Albania, Xhaxhiu et al. (2009), identified two species of ticks on the examined dogs represented by *Rh. sanguineus* (23.8%) and *I. ricinus* (0.6%). In Brasil, Silveira et al. (2009) collected a total number of 7318 ticks, and all of them belonged to *Rh. sanguineus*. Mathee et al. (2010) identified 11 species of ticks in examined dogs from Northern Cape Province in South Africa and 6 species in Namibia, the highest prevalence in both countries belonged to *Rh. sanguineus.*

In this study, the presence of a peak in april and one in september, has been observed, this aspect being related with the climate conditions of our country, these species of ticks favouring the biotops in our country. The results are closely related to similar studies developed in different European countries (Polland and Albania), with climate conditions such as our country (Zygner and Wedrychowichz, 2006; Xhaxhiu et al., 2009). In temperate climate areas, the biologica cycle of the ticks is characterised through active stages, in spring and autumn, when there is an increase in the density of the population, alternating with stages of hipobiosis (Mitrea, 2002).

The intensification of the infestation has registered relatively small values in the study shown here due to the fact that the animals have received antiparasitic treatments in their lifetime (fact from the history of the cases). Similar studies carried in different countries have registered variable results. So, Földvári and Farkas. (2005) identified a total number of 78 ticks per dog and Zygner et al. (2006) found a total number of 16 ticks per dog. The intensification of the tick parasitic infestation is influenced by climate conditions and biotope, which

assures the optimum parameters for the development of the biological cycle, adding the host's population density which has direct influence on the distribution of the tick population. All of these aspects will be reflected in the dynamic of the tick transmitted diseases.

## 3. CONCLUSIONS

3.1. The study shown here has revealed, on the body of dogs with babesios, two species of ticks, *Dermacentor reticulatus* and *Rhipicephalus sanguineus*, in different proportions.

3.2. Also, it has been observed that the two types of ticks have seasonal activity, with two peaks, one in the springtime and the other in autumn.

3.3. The obtained results impose the necessity of permanent surveillance of tick prevalence in dogs, and in other species, the role of those arthropodes in transmitting pathogen agents both in humans and animals is well known

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# SPECIES IDENTIFICATION THROUGH MITHOCONDRIAL DNA (MTDNA) ANALYSIS

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**Key words:** mtDNA, species identification

**SUMMARY**

Due to European regulations regarding intracomunitary meat and meat products commerce, as well as other animal products such as milk and milk products, eggs and egg products, animal furres, as well as increasing number of forensic cases regarding species identification, raises the need for developing and implementing specific and accurate techniques for biological samples analysis. One of the most powerful methods regarding accuracy, number of species that can be tested, as well as broad spectrum of biological samples that can be analyzed consist of mithocondrial DNA investigation by molecular techniques. This paper focuses on implementation of 12S ribozomal RNA sequence analysis on various mamalian and bird species.

Species identification from various biological samples has become an important tool in fields like forensic veterinary medicine, food industry and furres comercialization. Different methods have been used for this purpose, ranging from anatomical differences, histological examination of certain biological samples (like hair), particular proprieties of fat tissue, glycogen level in muscle tissue, protein and DNA analysis. Some of these methods have been reported to have different degree of limitations in practice, due to problems like specificity (glycogen level, histological differentiation, fat tissue analysis) arised from individual status like age, sex, habitats etc, breed and subbreeds for many domestic animals, complexity, requirements for baseline data regarding differences in protein composition and many more. Numerous analytical methods that rely on protein analysis have been developed for species identification, such as electrophoresis techniques (Kim *şi col, 1986, Skarpeid şi col,* 1998), immunoassays (Hsief *şi col,* 1998) and liquid chromatography (Ashmoore *şi col,* 1998). However, proteins are heat labile and might lose their biological proprieties. Moreover, their presence and characteristics depend on special cell types (Pfeiffer *şi col.,* 2004). Thus, for species identification, DNA analysis would be preferred over protein analysis.

Among the most commonly used techniques for DNA characterization, PCR-RFLP (*polymerase chain reaction - restriction fragment length polymorphism*) and sequencing are the most important. The first one is mainly applied for domestic animals, allowing differentiation of species from meat and meat products as well as other byproducts in food industry, whereas sequencing can be implemented in virtually every field that involves presence of genetic material.

## 1. MATERIALS AND METHODS

**Samples type**s subjected to the study was represented by various biological material (such as blood, brain homogenate, spleen, intestines) originated from different species of domestic and wild animals (Table 1). Tissue samples were initially homogenized using *MagNa Lyzer* instrument (*Roche Applied Science*), following manufacturer recommendation regarding speed and time, resulting in 10% organ suspension that was further centrifuged for supernatant collection.

**DNA extraction** was performed automatically using *MagNa Pure LC* Instrument (*Roche Applied Science*), with 200µl of previously obtained supernatants of the 10% organ suspensions or EDTA blood and elution in 100µl final volume

**PCR amplification** was performed using previously described primers (Kocher *şi col.,* 1989), generating a genomic product of 386 nucleotides from the small rRNA (12S rRNA) unit. The amplification was performed with *FastStart Taq DNA polymerase* (*Roche Applied Science*), following manufacturer recommendation, with a final reagents concentration of 0.6µM for primers, 2mM for MgCl2, 200µM of each dNTP and 2 units of enzyme. For each sample, 10µl of extracted DNA was used, with a final volume of 50µl per reaction

Samples were loaded on an *ICycler instrument* (*BioRad Laboratoires*) with the thermal profile consisted of 95ºC for 5 minutes for initial denaturation and enzyme activation, followed by 35 cycles of 95ºC for 35 seconds (denaturation), 50-53ºC for 35 seconds (annealing), 72ºC for 1 minute (extension), 7 minutes at 72ºC final extension and stored at 4ºC until gel loaded.

**Gel electrophoresis** was performed using 2% agarose gel stained with ethidium bromide and 1X TBE buffer (Tris Borate EDTA).

**Direct sequencing**: Specific amplicons bands were excised from the agarose gels and purified using *MinElute Gel Extraction Kit* (*Qiagen*), following manufacturer recommendation and amplified using *BigDye V 1.1 Cycle Sequencing Kit* (*Applied Bioscience*). Products were purified

using *CentriSep Spin Columns* (*Princeton, Applied Bioscience*) and loaded into a 3130, 4 capillary genetic analyzer.

**Dendrogram** was obtained with Mega 4 software, using NJ (Neighbour Joining) algorithm, Kimura 2 parameter and 3000 replicates bootstrap support (Figure 1).

***Table 1***

***species and biological material used***

| Species | Sample type | Species | Sample type |
|---|---|---|---|
| Goat | EDTA blood | Laboratory mouse | Brain, intestine |
| Sheep | EDTA blood | Dog | EDTA blood |
| Ren dear | EDTA blood | Cat | Brain |
| European bison | EDTA blood | Chicken | EDTA blood |
| Cow | EDTA blood | Red fox | Brain |
| Horse | EDTA blood | Wild boar | Spleen, EDTA blood |
| Domestic pig | Spleen, EDTA blood | Bear | Brain |

## 2. RESULTS AND DISCUSSION

Dendrogam obtained using 12SrDNA amplified fragment was clearely capable to distinguish between mamals and birds clases, as well as species identification within a specific family, with various degrees of accuracy, as follows:

1. within the large herbivores cathegory, the protocol was able to diferenciate between domestic and wild species of bovines from the three continents of Europe, North America and Asia;
2. different species of dear were clearely identified, including the romanian native species;

3. for small ruminants, dendrogam showed a clear distinction between sheep and goat species. Moreover, for each subdivision, amplified fragments had a considerably high degree of heterogeneity, thus allowing identification of different domestic and wild species;

4. *leporidae* family was clearely distinguishable from others and consequently different species from all over the world were identified;

5. regarding *suidae* family, unfortunately no clear distinction between wild and domestic native species was observed, thus, for this issue, different approaches might be needed;

6. *ursidae* family was also clearely identified, as well as the majority of the species that falls into this cathegory;

7. similary with small ruminants, the protocol was able to diferentiate between foxes and wild and domestic species of canine;

8. different species of mices and rats were also characterized, making this protocol suitable for exact species identification;

9. within the feline family, clear differences between large and small, domestic and wild as well as different habitats species was detected.

10. finally, regarding birds, important diferences between various domestic species were identified.

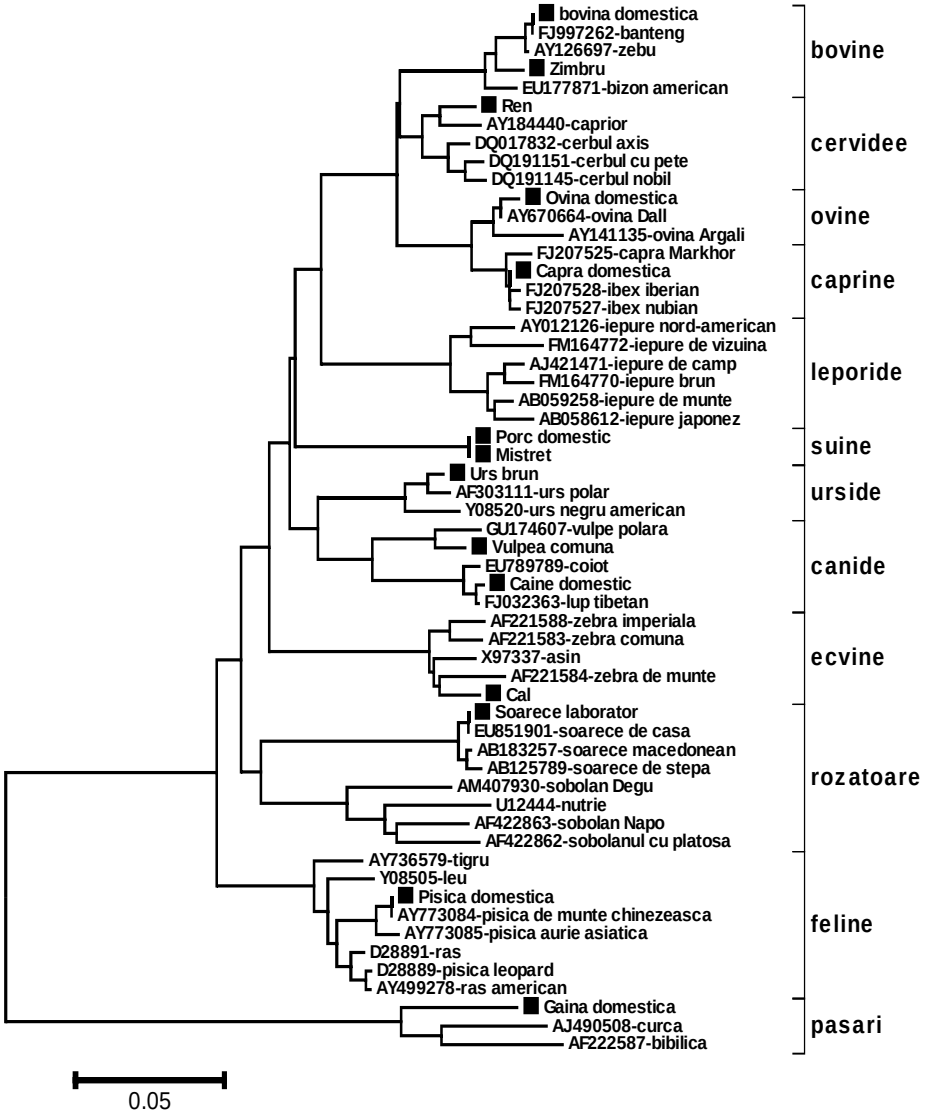


**Figure 1 – Dendrogram obtained using analyzed sequences (marked for easy recognition) and related sequences retrieved from GenBank**

## 3. CONCLUSION

Partial sequencing of small rRNA (12S rRNA) unit can be regarded as one of the most comprehensive method for species identification, being able (but not limited) to discriminate between mammalian and

avian classes, but also within the same genus for a vast number of domestic and wild species.

However, this technique can be applied only for one species at a time, meaning that it is not intended to discriminate between mixtures of different DNA species; therefore, in these cases, other methods should be applied (such as PCR-RFLP).

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# RESEARCHES REGARDING THE INCIDENCE OF CANINE PROSTATE DISEASES AND IT'S TREATMENT

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**Key words**: prostate, adenoma, chists, echography, dog.

**SUMMARY**

Now days, when the breeding of the pets has developed, from the growing interest of owners to obtain valuable offspring, male genital tract's pathology is of particular importance, and the prostate is an important and integral part of the pathology of male genitalia.
This research shows some of the casuistry of Obstetrics and Gynecology Clinic of the Faculty of Veterinary Medicine in Bucharest and is the presentation of 23 cases of prostate disease in dogs belonging to different races and with different ages.
This study aims to determine, based on cases under discussion, some prostate pathology parameters with reference to the dog. These parameters are represented by: frequency of illness, age groups affected, breeds prone to disease, best ways of treatment, the percentage of cured animals.
Most cases seen in our Clinic were found to breed Poodle, the average age affected was 13-14 years and the most common diseases were those of the prostate adenoma and prostate cyst.
Most cases were treated using antibiotherapy and osaterone acetate but we have treated several cases using surgical procedures too.
Most of the cases were treated, only 2 of 23 were lost by death.

The dog is certainly the most common domestic animal and it plays a significant role in Romanian society environment. An intimate involvement and cooperation so profound, not only in practice, but also in the emotional and affective plan, it is unprecedented in any other animal, even the horse.

At present, the breeding of company carnivores has developed, from the growing interest of owners to obtain valuable products, the male genital pathology is of particular importance, and the pathology of the prostate is one of its dominant.

For as early identification of prostate diseases in dogs and to achieve the most effective therapy is necessary to know them according to certain parameters: frequency of illness, age groups, affected breeds prone to disease.

With the objective tests, and with the help of effective diagnostic methods can get a pretty good percentage of healing of such conditions.

## 1. MATERIAL AND METHODS

Research has been conducted in the Clinic of Obstetrics, Gynecology and Andrology, in the Faculty of Veterinary Medicine from Bucharest in June 2009 - June 2010. During the research study were taken in 23 dogs with prostate disease, dogs belonging to different races and with different ages.

Each case was analyzed separately and the history was made, and there were ultrasound treatment and progress was recorded.

The main methods, by which prostate diseases were diagnosed and treated, were represented by: history, inspection, palpation, the assessment of body temperature, ultrasound and radiography.

A well-restored history, allows orientation toward the examination and diagnosis affected device, and there are times when history is sufficient for presumptive diagnosis.

The inspection was carried out in two stages: first there was an inspection at a distance (the distance), which was continued and completed the second stage with a close inspection (the approach position).

Goals in the palpation were related to the sensitivity of the prostate (tactile, thermal painful), rectal temperature, position, shape, size, integrity and consistency.

Ultrasound examination was performed with ultrasound Aquila Pro Vet, convex probe with frequency of 5 to 7.5 MHz and linear probe of 8-10 MHz multiple frequencies used for rectal examination. On ultrasound examination were followed ecodensity, ecostructure, homogeneity, size and profile of prostate contour.

The most important approach to the prostate was in the dorsal decubitus, the longitudinal and transversal approach, but in some cases the transrectal approach was used and linear probe utilized with a high frequency for exquisite images.

The diagnosis was based on clinical signs, clinical examination, laboratory and imaging.

The treatment was done by several methods depending on the diagnosis and prognosis.

The main methods of diagnosis and treatment were represented by: antiandrogenic therapy, orchidectomy, criptorhidectomy, marsupialization, omentalization and antibiotics.

As an antiandrogenic, the Osateron acetate was used, which is an inhibitor of androgen receptors correlated with the chemical action of progesterone and thus has antiandrogenic and progestogen effects. In

male dogs, it blocks the transport of the male hormone testosterone into the prostate. By inhibiting testosterone, reduces prostate size.

## 2. RESULTS AND DISCUSSIONS

The 23 cases included in the study covered many aspects of the disease, and presented many clinical forms. The most common were benign prostatic hyperplasia/hypertrophy, prostatic cyst, paraprostatic cyst, prostate abscess and polychistic prostate.

The studied animals were from several races, and age of the animals studied ranged from 7-14 years.

After the diagnosis was made and treatment was pursued in the specific case, the evolution was carefully followed.

Following ultrasound exam we had numerous images, of which we present the most representative in Fig. 1, 2, 3 and 4.

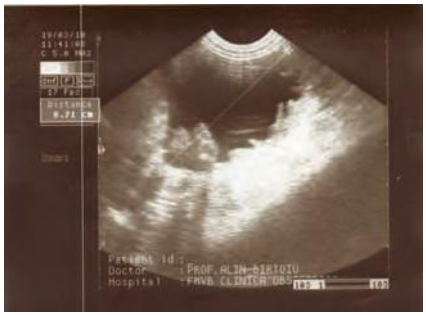

**Fig. 1. Prostate abscess (orig.)**

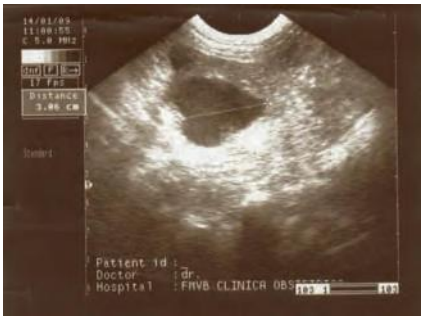

**Fig. 2. Prostate cyst (orig.)**

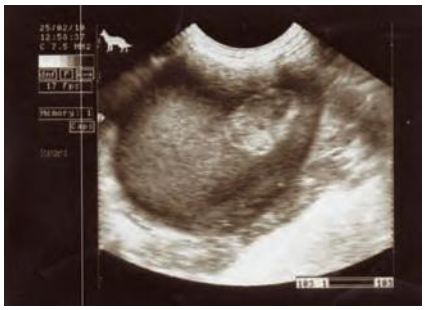

**Fig. 3. Giant prostate cyst and prostate tumor (orig.)**

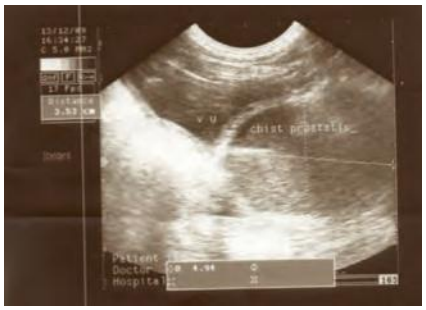

**Fig. 4. Paraprostatic cyst (orig.)**

Following the observation of cases we have discussed the following results. In our study the 23 cases examined were divided on a number of 13 different breeds.

***Table 1.***

**The incidence of prostate disease depending on breed**

| No. | Breed | No. of cases |
|---|---|---|
| 1. | Boxer | 3 |
| 2. | Caniche | 4 |
| 3. | Bichon Toy | 1 |
| 4. | Comună | 1 |
| 5. | Cocker | 2 |
| 6. | Dalmaţian | 1 |
| 7. | Ciobănesc German | 3 |
| 8. | Rotweiller | 2 |
| 9. | Setter | 1 |
| 10. | Pitbull | 1 |
| 11. | Doberman | 2 |
| 12. | Bull Terrier | 1 |
| 13. | Airdale Terrier | 1 |

In conclusion, most cases of male genital disorders were found in Poodle, respectively 4 cases (Table 1).

In our study the 23 examined cases were distributed in different age groups, from age 7 to age 13.

***Table 2.***

**The incidence of prostate disease depending on age**

| **Male age category** | **Case number** | **Case percent (%)** |
|---|---|---|
| 7-8 years | 2 | 8,69 |
| 9-10 years | 4 | 17,39 |
| 11-12 years | 8 | 34,78 |
| 13-14 years | 9 | 39,13 |

In conclusion, most cases of prostate disease were seen in 13-14 years age group, respectively 39.13%. It can be seen a trend of incidence of prostate disease morbidity with increasing age of the patients presented in our clinic.

In the 23 cases studied we have met both inflammatory, hormonal, metabolic and cancer.

***Table 3.***

**Types of diseases encountered in the prostate**

| No. | Types of diseases | Case number |
|---|---|---|
| 1. | Prostatic benign hypertrophy/hyperplasia, prostatic cyst | 5 |
| 2. | Prostatic cyst | 5 |
| 3. | Prostatic benign hypertrophy/hyperplasia | 1 |
| 4. | Tumor and paraprostatic cyst | 1 |
| 5. | Paraprostatic cyst | 4 |
| 6. | Prostatic abscess | 4 |
| 7. | Polychistic prostate | 3 |

In conclusion, most cases encountered in this study were those in which subjects were presented prostatic benign hypertrophy/hyperplasia associated with prostatic cyst, respectively 5 cases from a total of 23 cases and those of unique prostatic cyst, without being associated with prostatic benign hypertrophy/hyperplasia. At the opposite end there are the cases of prostatic benign hypertrophy/hyperplasia as a single morbid entity and prostate tumor.

***4. Types of treatments*** applied in cases of prostate disorders studied in the Department of Obstetrics and Gynecology.

In some cases the only recourse is based on antibiotic therapy or antiandrogenic, but in most cases surgery was necessary using omentalization, marsupialization or simply bilateral orchidectomy.

***Table 4.***

**Types of treatments used in diseases of the prostate**

| No. | Types of diseases | Treatment | Resolution moment | Maintenance of the resolution in time |
|---|---|---|---|---|
| 1. | Prostatic benign hypertrophy/hyperplasia, prostatic cyst | Osateron acetat | 16 days | 120 days |
| | | Osateron acetat and orchidectomy | 80 days | permanently |
| | | Osateron acetat | 30 days | 270 days |
| | | Osateron acetat | 18 days | 210 days |
| | | Osateron acetat | 24 days | 135 days |
| 2. | Prostatic cyst | Osateron acetat | 14 days | 180 days |
| | | Osateron acetat | 20 days | 240 days |

| No. | Types of diseases | Treatment | Resolution moment | Maintenance of the resolution in time |
|---|---|---|---|---|
| | | Antibiotherapy and omentalization | 25 days | permanently |
| | | Osateron acetat | 30 days | 180 days |
| | | Osateron acetat | 45 days | 270 days |
| 3. | Prostatic benign hypertrophy/hyperplasia | Osateron acetat | 21 days | 210 days |
| 4. | Tumor and paraprostatic cyst | Antibiotherapy and omentalization | 45 days | permanently |
| 5. | Paraprostatic cyst | Antibiotherapy and marsupialization | deceased | deceased |
| | | Antibiotherapy and marsupialization | 70 days | permanently |
| | | Antibiotherapy and omentalization | deceased | deceased |
| | | Antibiotherapy and omentalization | 60 days | permanently |
| 6. | Prostatic abscess | Antibiotherapy and marsupialization | 45 days | permanently |
| | | Antibiotherapy and marsupialization | 35 days | permanently |
| | | Antibiotherapy | 21 days | permanently |
| | | Antibiotherapy and marsupialization | 45 days | permanently |
| 7. | Polychistic prostate | Osateron acetat | 65 days | 180 days |
| | | Osateron acetat | 90 days | 240 days |
| | | Osateron acetat and orchidectomy | 30 days | permanently |

In case of prostatic benign hypertrophy/hyperplasia associated with prostatic cyst, 4 of 5 subjects were treated with osateron acetate, and in the fifth subject orchidectomy was used as adjunctive therapy. Remission of clinical signs after treatment with osateron acetate, started 22 days (range between 16 and 30 days) and persisted for an average of

183.75 days (range 120 days to 270 days). In combination with orchidectomy, the onset of disease remission occurred in 25 days but the delivery was final.

In cases of prostatic cysts, 4 of 5 subjects were treated with osateron acetate, and in the fifth subject antibiotics and omentalization were used as basic method. After treatment with osateron acetate, remission of clinical signs began at 27.25 days (range between 14 and 45 days) and persisted for an average of 217.5 days (range 180 days to 270 days). In omentalization combined with the antibiotic the disease began to reverse after 80 days but it was final.

The only case with prostatic benign hypertrophy/hyperplasia was treated with osateron acetate. Onset of remission occurred in 21 days and remained in complete remission status for 210 days.

The subject who showed the tumor and paraprostatic cyst was treated using omentalization in association with antibiotic. Remission of disease began at 45 days post treatment and was finally installed.

In case of paraprostatic cyst, 2 of 4 subjects were treated with antibiotics and marsupialization and the other two subjects were treated with antibiotics and omentalization as the basic method. After treatment with antibiotics and marsupialization, remission of clinical signs started 70 days and remained permanently in one case, the second dying. Omentalization in combination with antibioticsmade the disease began to reverse after 60 days but was permanent in one case, the second dying.

In case of prostatic abscess, 3 of 4 subjects were treated with antibiotics and marsupialization and the other subject was used only as a means of basic antibiotics. After treatment with antibiotics and marsupialization, remission of clinical signs began at 41.66 days on average (between 35-45 days) and remained permanently in one case while the case treated only with antibiotics, the disease resolved after 21 days, remaining permanently.

Two of three cases of polycystic prostate were treated with osateron acetate, the third with osateron acetate and orchidectomy. In cases treated with osateron acetate remission started on average 77.5 days (65-90 days) and maintained an average of 210 days (180-240 days). Osateron acetate in combination with orchidectomy, remission occurred after 30 days and was finally installed.

In conclusion, in most cases the administration of antiandrogens were used. At the opposite end stands the treatment only with antibiotics.

***5. Number of cases*** that have developed positively compared to the number of cases had a negative outcome.

In our study, two cases (8.69%) of 23 examined cases had a poor outcome, and the remaining 21 cases (91.31%) showed a favorable trend.

***Table 5.***

**Evolution seen in prostate disease**

| No. | Evolution | Number of cases | Percent |
|---|---|---|---|
| 1. | positive | 21 | 91,31% |
| 2. | negative | 2 | 8,69% |

In conclusion, most of the cases ended favorably (21, 91.31% respectively).

## 3. CONCLUSIONS

3.1. In our study the 23 cases examined were divided on a number of 13 different breeds.

3.2. In our study the 23 examined cases were distributed in different age groups, from age 7 to age 13.

3.3. Most cases of prostate disease were seen in 13-14 years age group, respectively 39.13%.

3.4. Most cases encountered in this study were those in which subjects were presented prostatic benign hypertrophy/hyperplasia associated with prostatic cyst, respectively 5 cases from a total of 23 cases and those of unique prostatic cyst, without being associated with prostatic benign hypertrophy/hyperplasia. At the opposite end there are the cases of prostatic benign hypertrophy/hyperplasia as a single morbid entity and prostate tumor.

3.5. In most cases the administration of antiandrogens were used. At the opposite end stands the treatment only with antibiotics.

3.6 Two cases (8.69%) of 23 examined cases had a poor outcome, and the remaining 21 cases (91.31%) showed a favorable trend.

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# COMPARATIVE STUDY OF THE PELVIC LIMB SKELETON IN THE CAT *(FELIS CATUS)* AND IN THE TIGER *(PANTHERA TIGRIS)*

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**Key words:** skeleton, pelvic limb, cat, tiger.

**SUMMARY**

Our studies have aimed to make a comparative description of the pelvic limb skeleton in the domestic cat (*Felis catus*) and in the tiger (*Panthera tigris*). The biologic material consisted of 2 tiger cadavers provided by the Turda Zoo, Cluj County, and osseous pieces form 6 cat bodies. The osseous pieces were processes in the Comparative Anatomy Laboratory of the Faculty of Veterinary Medicine Cluj-Napoca.

After comparing the pelvic limb skeletons of these two species, we have concluded that the differences between the two are mainly found in the pelvic zonoskeleton and zeugopodium regions, and to a lesser extent in the stylopodium and the autopodium region (the latter referring mostly to the metatarsal bones). These differences are correlated with the body mass, the muscle mass and the living style of the cat compared to those of the tiger.

The domestic cat (*Felis catus*), smallest of the feline family, is a highly appreciated pet and thus occupies an important place in the anatomy literature worldwide, though most descriptions are made by comparison to the dog, carnivore belonging to another taxonomic family.

The tiger (*Panthera tigris*) is the biggest feline and a wild predator living in different habitats from Asia. It is rarely found in both our local and international anatomy literature.

Wishing to expand the morphological database on felines in general, and on the tiger in particular, we have started a comparative study of the two feline's skeletons, limited in this paper to the pelvic limb. The data obtained by us can facilitate the recognition of species based on skeleton characteristics and the study of correlations between muscles development, bone surface aspects and life style of these animals.

## 1. MATERIAL AND METHOD

To make this study, we have used as biological material 2 tiger bodies, males, 8 to 10 years of age, offered by the Turda Zoo, Cluj County, and 6 cat bodies, adults, common European breed, of which 4 females and 2 males. The cadavers were processed in the Comparative Anatomy Laboratory of the Faculty of Veterinary Medicine, Cluj-Napoca.

The first stage of the process consisted in the removal of the musculo-tendinous tissues and in isolating the anatomic regions of the pelvic limb. The parts thus obtained were thermically processed in several stages: I - 5 hours boiling, followed by another removal of the soft tissues, II - 3 hours boiling, in a solution of surface active agents, repeated for those osseous pieces which required further cleaning. The last stage was a treatment of the bones with bleaching agents. After the completion of the preparation, the bones were sorted on anatomical regions, were examined and all the anatomical elements of their surfaces were identified, along with the major differences between the two species.

## 2. RESULTS AND DISCUSSION

### Pelvic Zonoskeleton

The **coxal bone** is an osseous complex which joins its symmetric companion to offer a solid base for the body's support and propulsion by the hind limbs. Both coxal bones articulate with the sacrum to form the pelvic cavity.

Viewed as a whole, in both species, the pelvis has an overall elongated shape and an almost horizontal positioning (Gheție and Hillenbrand, 1971). Compared to its sagittal diameter, the transverse diameter of the pelvis is bigger in the domestic cat than in the tiger, thus the pelvic cavity is wider in the former (Fig.1). The ischial arch also has a wider opening in the cat. In this species *obturator foramen* is large (Barone, 1966) compared to the one in the tiger's coxal bone, and its longitudinal diameter is inclined latero-cranially, while in the tiger it is almost parallel to the median plane (Fig.1). The cavity of the acetabulum does not present notable differences between the two species, but the *spina ischiadica* is much more prominent and rugged in the tiger (Fig.2).

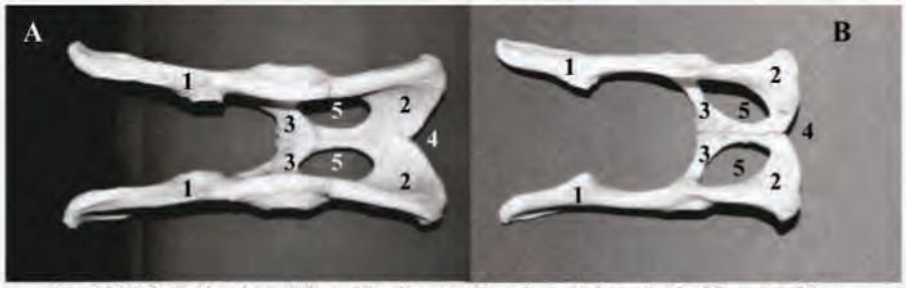


**Fig.1.**Pelvis in the tiger (A) and in the cat (B) – dorsal view: 1 - ischium, 2 - ilium, 3 - pubis, 4 - ischiatic arch, 5 - *obturator foramen*.

The **ilium** makes up the cranial part of the coxal bone, and is composed of a wing and a body. In both species, the iliac wing is rectangular with smooth angles and is less tilted laterally than in other species (Damian *et al.*, 2001). Its borders are rounder in the cat, while in the tiger they are sharp and rugged. Its lateral face presents a smooth and prominent dorsal gluteal line in both species, delimiting the dorsal border of the external iliac fossa (Barone, 1966), which is deeper in the tiger than in the cat (Fig.2). The medial face displays an articular surface for the sacrum - *facies auricularis* - placed near the caudal margin, generally shaped as the letter C, with individual variations in both species. In the tiger, the margins of this surface are better highlighted. The body of the ilium is flattened latero-laterally. The greater sciatic notch is thick and concave in both species. The iliopectineal crest presents a psoadic tubercle which is more rugged in the tiger (Fig.2).

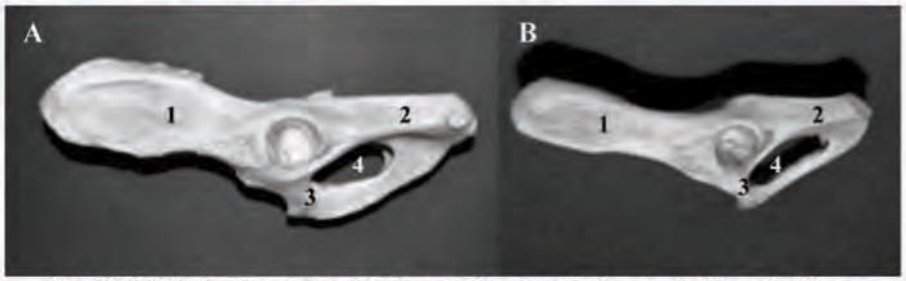


**Fig.2.**Pelvis in the tiger (A) and in the cat (B) – lateral view: 1 - ischium, 2 - ilium, 3 - pubis, 4 - cavity of the acetabulum.

The **ischium** and the **pubis,** together with the ilium, take part in forming the cavity of the acetabulum, and the first two form the floor of the pelvic cavity. The body of the ischium is wider in the cat, while in the tiger the tuberosity of the ischium, also known as the point of the buttock, has a

much more rugged surface. The transversal arm of the pubis is much more thin and elongated in the cat (Fig.1). The ischio-pubic symphysis has a convex profile, thicker and with a rougher surface in the tiger and rectilinear in the cat (Fig.2).

**Pelvic Stylopodium**

The **femur** is the anatomical base of the thigh. It is a long bone, with a ventro-cranially tilted position. The diaphysis is rectilinear and has convex surfaces in both species. It is proportionally longer in the cat than in the tiger (Fig.3). In the domestic cat, the caudal face of the diaphysis is separated from the lateral face by a longitudinal crest which continues the greater trochanter mare. In the tiger, this crest is better highlighted and rugous, and the *linea aspera* is much more prominent (Fig.4).

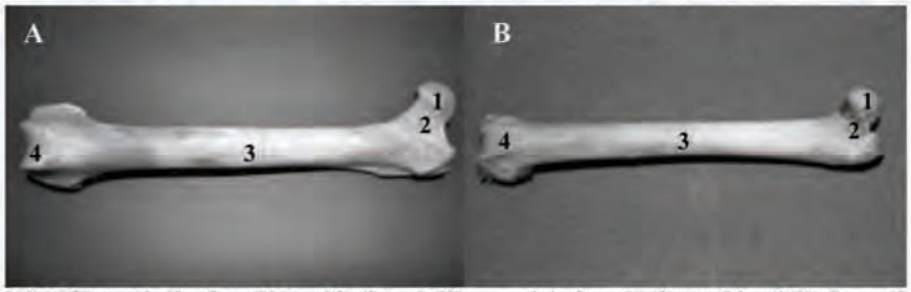


**Fig.3.**Femur in the tiger (A) and in the cat (B) – cranial view: 1 - femoral head, 2 - femoral neck, 3 - diaphysis, 4 - trochlea.

The proximal epiphysis is very much alike in both species, aside from a slightly longer femoral neck and the two trochanters with more irregular and rugged surface in the tiger (Fig.4).

There are also few differences in the distal epiphysis region: the trochlea (patellar surface) is more prolonged on the cranial surface of the

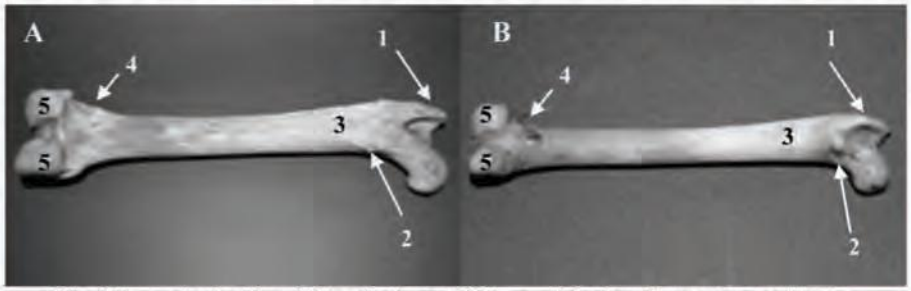


**Fig.4.**Femur in the tiger (A) an in the cat (B) – caudal view: 1 - greater trochanter, 2 - lesser trochanter, 3 - *linea aspera*, 4 - supracondyloid tubercle, 5 - condyles.

diaphysis in the cat, and the supracondyloid tubercle (plantar tubercle) is more prominent and rugged in the tiger, while in the cat it is reduced, often even leveled out (Fig.3 and Fig.4). Both species present a pair of **femoral sesamoids** articulated with the dorsal sides of the condyles.

In both species, the **patella** is a small oval bone whose smooth caudal surface articulates with the femoral trochlea. This surface is almost circular and clearly separated from the inferior, rounded, rough and riddled apex, in the tiger, and in the cat the whole cranial surface is smooth, without a clear distinction between this two elements. Its cranial side is the insertion site for the cranial muscles of the thigh, whose action is transmitted to the tibia through the patellar ligaments (Barone, 1966). This face is rugged in its inferior side the tiger and uniformly smooth in the cat (Fig.5).

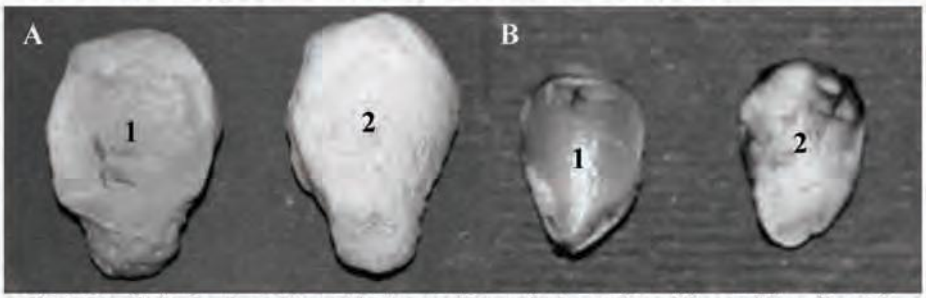


**Fig.5.**Patella in the tiger (A) and in the cat (B): articular surface (1), cranial surface (2).

**Pelvic zeugopodium**

The **tibia** and the **fibula** are the anatomic base of the calf. Both in the cat and in the tiger, these ventro-caudally tilted bones articulate with one another only at the level of the epiphyses, the interosseous space being thus visible on the whole length of the diaphysis, larger in the proximal half (Fig.6) (Coțofan *et al.*, 1999).

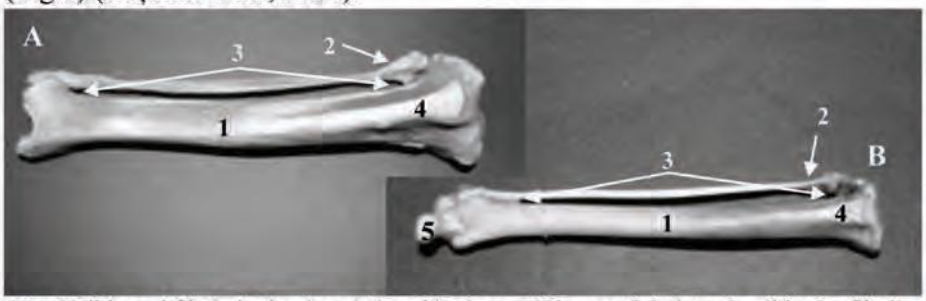


**Fig.6.**Tibia and fibula in the tiger (A) and in the cat (B) – cranial view: 1 - tibia, 2 - fibula, 3 - interosseous space, 4 - tibial crest, 5 - tarsal bones attached to the tibia.

The tibia is the main bone of the calf. As a whole, it has a longer diaphysis in cats than in tigers (Fig.6). In the latter, though, the medial face

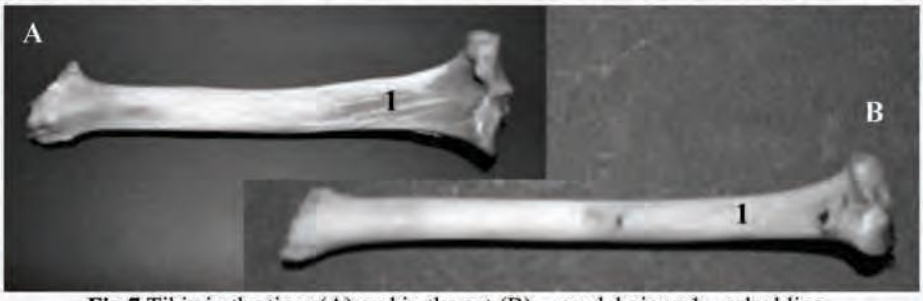


**Fig.7.**Tibia in the tiger (A) and in the cat (B) – caudal view: 1 - soleal line.

of the tibia is much more rugous and the soleal line of the caudal face is deeper with sharp edges (Fig.7). The tibial crest is thicker and more prominent in the tiger (Fig.6). The epiphyses of the tibia in both species are similar, but in the tiger, the cochlea of the distal epiphysis has a more pronounced obliquity relative to the longitudinal axis of the bone.

The fibula is much thinner than the tibia, but it's completely developed in both species (Coțofan *et al.*, 1999). Similar to the tibia, the fibular diaphysis is longer and smoother in the cat and irregular and with sharp interosseous and caudal crests in the tiger (Fig.6).

**Pelvic autopodium**

The **tarsus** is the anatomical base of the hock region and is present in the complete formula of seven tarsal bones in both species. They are placed on two rows: a proximal one, made up of the calcaneus and the talus, and a distal one, made up of the navicular, the cuboid and three cuneiform bones (lateral, intermediate and medial) (Fig.8A1 and Fig.8A2) (Barone, 1966).

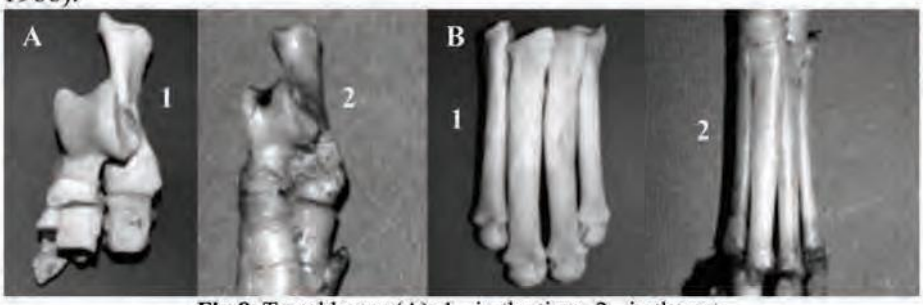


**Fig.8.** Tarsal bones (A): 1 - in the tiger, 2 - in the cat.
Metatarsal bones (B): 1 - in the tiger, 2 - in the cat.

In both species, the hind limbs have only four toes, which accounts for them having only four **metatarsal** bones each; the first metatarsal bone is always rudimentary. These are long bones, with the third and the fourth metatarsal bones being the best developed in both species (Fig.8B1 and Fig. 8B2) (Gheție and Hillenbrand, 1971). These bones also present on their proximal epiphyses lateral prominences which help them lean each on the next one. It is also notable that related to the whole hind limb skeleton, the cat's metatarsal bones are longer than the tiger's.

The four toes of the hind limb of both species are each made up of three **phalanges** and a pair of **sesamoid bones** placed caudally to the metatarsophalangeal joint (Fig. 9A and Fig.9B) (Barone, 1966). In the cat, as well as in the tiger, the retractile nature of the claws determines a singularity in the distal phalanges, which are placed almost vertically when at rest.

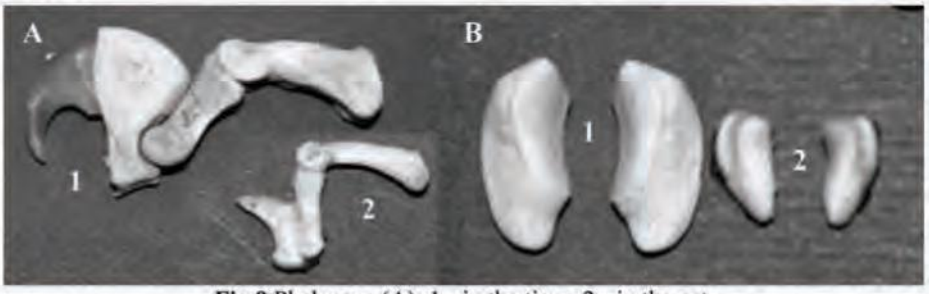


**Fig.9.**Phalanges (A): 1 - in the tiger, 2 - in the cat.
Sesamoid bones (B): 1 - in the tiger, 2 - in the cat.

When comparing the general aspect of the two hind limb skeletons, it is noticeable that bones are smoother and longer in cats, corresponding to a small body mass and to a finer muscle mass, which allow greater agility in vertical jumps and in climbing, in accordance with this species' accommodation to hunting smaller sized prey.

In tigers, the bones of the hind limb are robust, compact, with rough surfaces which allow strong anchorage for a well developed muscle mass and support for an important body mass. All these features provide the appropriate force for hunting down prey that is even larger than its hunter and the adaptability to a large range of terrain types, but with a life-style closer to the ground. These special morphologic developmental features are in accordance with the new tridimensional geometrical evaluations of bone allometry offered by computed tomography (Doube *et al.*, 2009).

## 3. CONCLUSIONS

3.1. The coxal bones make up a pelvis which is slightly larger in the cat than in the tiger, but which is elongated and almost horizontally placed in both species.

3.2. The *obturator foramen* is larger in cats, and the ischiatic arch also has a wider opening in this species.

3.3. The femur doesn't have any important differences between the two species, but, in the tiger, *linea aspera,* the crest on the caudal face, the two trochanters and the plantar tubercle are more rugged and more prominent.

3.4. The tibia and the fibula are more elongated in the cat. In tigers, the medial face of the tibia is rough and the caudal face presents a much deeper soleal line; the fibula has sharper interosseous and caudal crests, and the cochlea of the distal epiphysis has a more pronounced obliquity.

3.5. The autopodium is similar in the two species, comprising seven tarsal bones, four metatarsal bones, which are longer in the cat, and four toes made up of three phalanges and a pair of sesamoid bones each, with a singular positioning of the distal phalanges because of the presence of retractile claws.

3.6. Considering the hind limb skeleton as a whole, we have noticed that the domestic cat has longer and smoother bones, corresponding to its fine muscle mass and remarkable mobility.

3.7. In the tiger, the bones of the pelvic limb are more robust, with rougher surfaces which allow anchorage of a strong muscle mass, in accordance with the increased body mass and remarkable force of this wild feline.

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