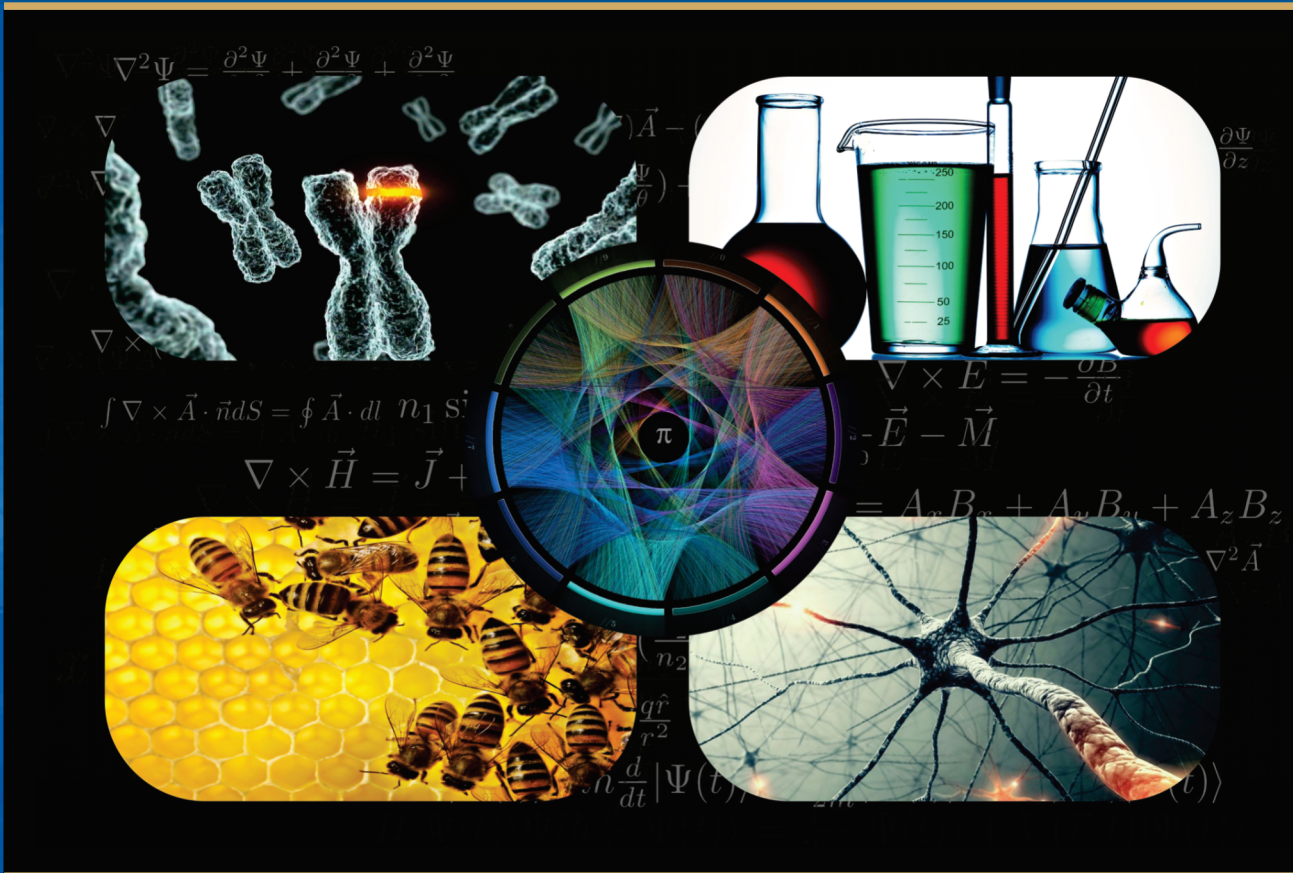


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Phylogenetic Relationships of some Wild and Cultivated Barley Accessions Using Seed Storage Protein Markers

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Abstract

Barley is of renewed interest throughout the world because of its nutritional value and low glycemic index. It has been suggested that biochemical accomplished with molecular markers can be used to solve population diversity of barley. Hordeins are the storage proteins of the wild and cultivated barley samples. For this reason, we analyzed genetic diversity in the storage protein hordein encoded loci in seeds from 106 accessions of wild and cultivated of barley accessions from different countries. Cluster analysis by using Nei genetic distance and UPGMA methods all studied wild and cultivated barley accession divided into 6 main groups. Grouping of Azerbaijan wild and cultivated barley accession in the same cluster showed that Azerbaijan cultivated barley accessions was clearly distinguished from its wild accessions. The presence of high level of diversity among the tested genotypes grouped into divergent clusters indicated their suitability for further research can be done in this direction by selecting superior barley genotypes.

Keywords: wild and cultivated barley, seed storage protein, phylogenetic relationships

Introduction

Cereals such as wheat, rice, barley, maize, and oats account for the majority of agricultural output. Barley (*Hordeum vulgare* L.) is the world's fourth most widely cultivated grain and one of the top ten agricultural plants. It is used in the production of food, feed, and malt. Barley has strong resilience to abiotic stresses, and in order to conserve genetic variety and reduce genetic loss, we must preserve and identify genetic diversity in both wild and cultivated barley.

Storage proteins in barley grains are categorized into two groups based on their solubility: globulins and prolamins (Gubatz S, Shewry PR, 2011). The prolamins found in barley grains are known as hordeins, and they are only found in the cells of

the starchy endosperm (Yupsanis T, 1990) & (Shewry P.R., 1993). Hordeins, the main storage proteins in barley seed and it contain 35 to 50% nitrogen of whole seed. It shows significant inter-genotypic variability and have been used as cultivar identification markers in studies of genetic diversity in collections (Doll H. and Brown A.H.D., 1979) & (Shewry P.R., 1983) & (Shewry P.R. and Milfin B.J., 1982) & (Heisel S.E., 1986.)& (Vapa, 1996). Hordeins were classified into four groups in polypeptide families (A, B, C, and D) based on electrophoretic mobility in sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid composition (Shewry P.R. and Milfin B.J., 1985).

Monomeric prolamins (according to wheat gliadins) benefit from a high level of diversity in both cultivated and wild barley species (Pan ZF, 2007). These proteins can be classified as α , β , γ , and ω areas (Eshghi & Akhundova, 2009).

The whole hordein map, contains a large number of alleles, may be utilized as a fundamental technique to distinguish cultivars, identify barley seeds, and determine heritability, as well as breeding programs.

The aim of this work was to explore the phylogenetic relationships of wild and cultivated barley accessions using biochemical markers as hordeins as a first step towards their further utilization in breeding program.

Materials and methods

In this study, 106 wild and cultivated barley accessions from different countries which had been

provided by National Azerbaijan Genbank were investigated, focusing on phylogenetic analysis in hordein. For extraction, electrophoresis and identification of hordein areas, (Poperelya and Mujarinko's., 2001) method was used. Hordeins were extracted from mature grains with 0.25 ml solution containing 6.9% acetic acid and 5% 2-mercaptoetanol and 16% urea and 0.01% pyronine. 0 and 1 coefficients were calculated for all the genotypes, depending on the presence (1) or absence (0) of the bands. It was also used in obtaining other results as well as similarity coefficients matrix of Nei (1978). Furthermore, in order to classify the accessions, cluster analysis was done using UPGMA (Unweighted Pair Group Method with Arithmetic Means). Calculating similarity coefficients matrix and dendrogram was done by PowerMarker program.

Result and discussion

The genetic distance between parents is directly proportional to the extent of heterosis found in progenies. Experiments on genetic variability provide a base of information regarding trait wise variation in the experimental material (Cheres et al., 2000).

Figure 1 shows a dendrogram obtained using the UPGMA method based on the Nei (1978) genetic distance index. Through cluster analysis, 106 genotypes from 15 barley populations included in the study were divided into 6 main groups based on hordein. The first cluster consist of 22 cultivated and wild barley samples from Azerbaijani origin. The placing of cultivated and wild accessions within a cluster is proof that Azerbaijani barley genotypes are composed of its ancestral wild relatives. At the same time, in the current group, genotypes 37 with 33, 41 with 40 and 39, 32 with 24, 28, and 7, 47 with 46 and 42, 45 with 44 and 43 were completely identical based on hordein proteins. In other words, the current samples were not identified from each other on the base of hordein. The second cluster also consisted of only 12 barley samples originated Azerbaijan. In this group, genotypes 29 and 26, 23 and 16, 20 and 21, 38 and 31 and 30 were found to be genetically similar. The third cluster consists of genotypes from France and Germany. Samples 61, 62, 63, 64 and 65 of French origin were identical in this group. Most of the barley genotypes studied are grouped in the 4th cluster. The barley samples from Germany, Czech, Kazakhstan, Poland, USA, Swiss and Russian were grouped in this cluster. Placing of Europe origin barley samples in the current group is reminiscent of the gene flow between these countries. The fifth cluster consisted of barley samples No. 73, 74 and 75 from Denmark, No. 79, 80, 81 and 82 from Georgian, No. 104, 105 and 106 from Canada and No. 72 from Czech Republic. The 6th cluster consists of cultivated barley samples of originated from Azerbaijani, Turkish and Hungary.

The cluster analysis revealed that considerable variation existed among genotypes that could be implicated in selection of wheat for the development or improvement of cultivars and germplasm.

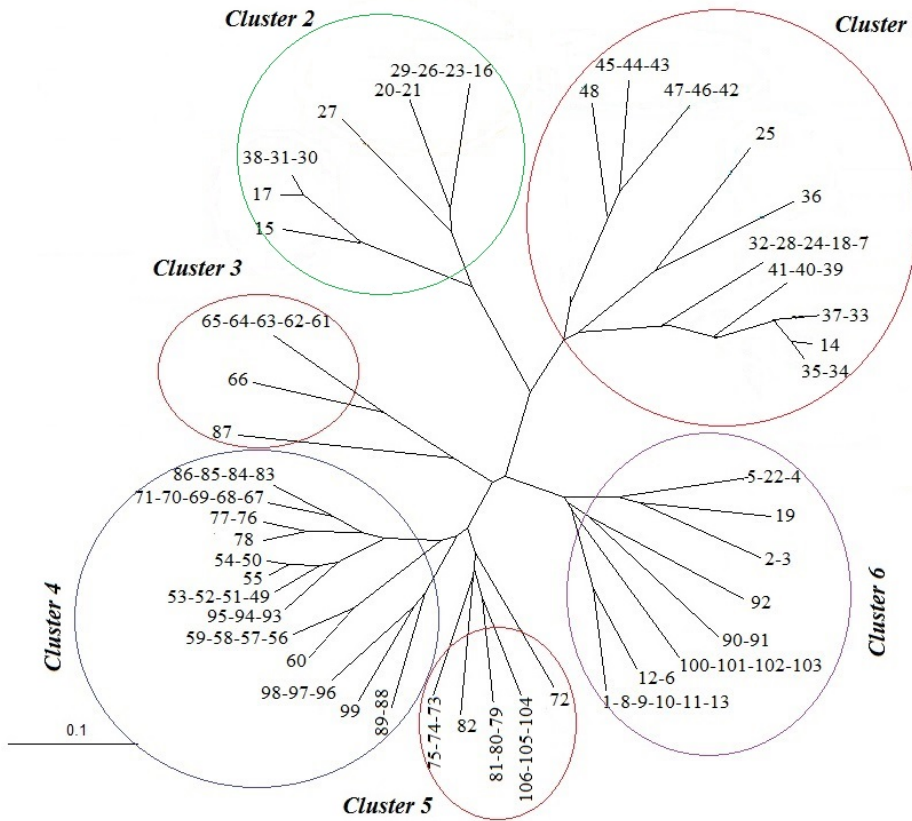


Figure 1. Dendrogram with Nei genetic distance of 106 barley accessions from different countries

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Effect of Cultivation Methods on Number of Plants of Winter Wheat Varieties After Wintering in Dryland Condition of Southern Mughan

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Abstract

The article shows results of the study about the effect of cultivation methods on the number of winter wheat plants after wintering in dryland condition. So, in a 3 factor (2x3x3) field experiment, chickpea, winter bread and durum wheat varieties were selected as predecessors and no-fertilizers, N₆₀P₆₀ +10 t manure and N₉₀P₆₀K₄₅ norms were used in each three cultivation variants which included: 1. Ordinary (ploughing at a depth of 20-22 cm + disking + harrowing; 2. Double disking at a depth of 10-12 cm with heavy disk harrow; 3. One disking at a depth of 10-12 cm with heavy disk harrow. Number of wintering plants and field germination (%) were found by counting in marked in 4 different points of the field for each variant.

Plants were counted again after wintering, at tillering stage of plants, in the areas of the field marked in each variant and the number of plants surviving in winter was defined according to field germination. The winter hardness of the varieties, after different predecessors, soil cultivation and root nutrition according to the number of wintering plants. The number of wintering plants was higher at predecessor of chickpea; also, the results were higher by double disking of the soil with heavy disk harrow at a depth of 10-12 cm. The highest result was obtained at the background of N₆₀P₆₀ +10 t manure.

It was found that by double disking of the soil with heavy disk harrow at a 10-12 cm depth, the winter hardness of plants was higher at both predecessors, cultivation in the background of N₆₀P₆₀ +10 t manure.

Keywords: dryland, soil cultivation, predecessors, nutrition condition, winter hardness

Introduction

The development of the agricultural sector, which is one of the main sectors of the non-oil sector, is one of the most important issues to ensure food security in the country. At present, the living standards and the stuff required for the well-being of population depends on the development of agriculture.

Therefore, the main task facing agrarian science in terms of grain production is to fully meet the needs of population for grain and grain products by producing higher yields from a single sown area.

Sustainable and dynamic development of production in agriculture, maintenance and improvement of soil fertility, increasing crop productivity and quality are impossible without the application of proper cultivation methods (Aslanov & Aslanov, 2014; Cherkasov, & Pykhtin, 2006).

The reason why the income from winter grains is lower than that of many agricultural crops is that the obtained yield is lower than the cost incurred for cultivation of the plants (Rzayev & Ismayilov, 2010). One of the main indicators of productivity is the winter hardiness of plants.

This indicator depends on the biological characteristics of the variety, the cultivation methods used and the environmental conditions. Thus, the choice of proper cultivation methods in specific agro-ecological conditions, on the one hand, leads to a certain increase in winter hardiness of plants, on the other hand, the minimization of cultivation by the application of energy-saving technologies which protect the soil from degradation to some extent and provides conditions to increase economic efficiency.

Materials and methods

Productivity of agricultural crops is one of the main indicators in the farming system (Aliyev, 2015). As cultivation methods have a direct impact on the viability of plants, which is one of the main indicators of productivity, it is possible to increase productivity through their proper selection.

In order to study the above-mentioned issues, a field experiment was conducted by planting winter wheat varieties after predecessors, with application of soil cultivation and nutritional conditions at the Jalilabad Regional Experimental Station (RES) of the Research Institute of Crop Husbandry, in the dry conditions of South Mugan.

Different subtypes of main gray-brown (brown) soils predominate in the region (Mammadov, 2007). According to the average multiplicity, the average annual

temperature is 14.10C, and the total active temperature during the growing season is 4300-4400 degree. The number of frost-free days is 250-280 days, and the amount of precipitation varies from north to south between 300 and 450 mm. Rainfall is unevenly distributed throughout the year and it rains mainly in spring and autumn months.

The three-factor (2x3x3) field experiment is based on the following scheme:

Factor A - predecessor: winter wheat, peas;

Factor B - method of cultivation: traditional cultivation (plowing at a depth of 20-22 cm + discing + leveling), 2 times discing with a heavy vertical trowel at a depth of 10-12 cm, 1 plowing with a heavy disc trowel at a depth of 10-12 cm;

Factor C - nutritional conditions: without fertilizer, N₆₀P₆₀ + 10 tons of manure, N₉₀P₆₀K₄₅.

The experimental area consists of three cultivation options for each predecessor, and each cultivation variant was divided into 3 spots with 50.4 m² area (3.6m x 14m) and 0.6 m distance between them. The experiment was performed in 4 repetitions with 4 m between cultivars, 3 m between varieties and 2 m between repeated plantings. The durum wheat variety "Barakatli 95" and the soft wheat "Gobustan" were studied using 3 fertilizer norms in each cultivation variant.

The number of plants surviving in winter was determined in the first decade of March by counting in two rows with a length of 83.3 cm (4 x 83.3 cm x 30 cm), ie in 4 places marked for each variant in the area of 1 m² (Musayev et al., 2008).

Results and discussion

One of the indicators of productivity is the amount of plants which survive in winter. The fact that plants spend the winter in optimal conditions is due to the correct choice of basic cultivation methods. Thus, the winter hardiness of plants depends not only on the characteristics of the variety, but also on the cultivation factors. Hence, a wide range of reaserches have been conducted in this regard throughout the country.

Aliyev A.M. notes that the correct selection of the predecessor and optimal nutritional conditions have a positive effect on the overwintering of plants. This is reflected in the growth of production (Aliyev, 2013). Also Ahmadov Sh.H., in his research, found that with the use of optimal nutrition, the number of plants that survived the winter increases and this leads to an increase in the rate of productivity (Ahmadov, 2014).

In our three-factor (2x3x3) field experiment which was to study the effect of soil cultivation and nutritional conditions on winter hardiness of plants, after different predecessors, the amount of plants that survived through winter was calculated according to the methodology and the variance analysis of the two-year results (average for 2019-2020) is given in Tables 1 and 2.

Thus, according to the two-year average results in Barakatli-95 wheat variety, the number of plants survived in winter was 204 in 1 m² area by applying traditional cultivation, 275 by 2 times discing of soil with a heavy trowel at a depth of 10-12 cm and 242 by one time discing of soil with a heavy trowel at a depth of 10-12 cm.

After its pea predecessor, this indicator was estimated to be 223, 299 and 268, respectively. At nutritional conditions, high indicators were observed for each predecessor, in the variant of N₆₀P₆₀ + 10 t of manure, so, this has been respectively 228-249 by using traditional cultivation, 300-332 by 2 times discing of soil with a heavy trowel at a depth of 10-12 cm and 265-299 by one time discing of soil with a heavy trowel at a depth of 10-12 cm.

These indicators were relatively high in "Gobustan" wheat variety. Thus, after its predecessors of wheat and peas, in traditional cultivation, this varied between 271-301, it was 367-391 by 2 times discing of soil with a heavy disc trowel at a depth of 10-12 cm, and 324-360 by one time discing of soil with a heavy trowel at a depth of 10-12 cm. A relatively high rate was observed by the use of N₆₀P₆₀ + 10 t of manure after both predecessors. In this condition, the highest rate was 434, after the pea predecessor, by 2 times discing of soil with a heavy disc trowel at a depth of 10-12 cm.

Table 1. Results of three-factor variance analysis of the effect of predecessor, soil cultivation and nutritional conditions on the amount of winter-surviving plants belonging to winter wheat varieties

(Average for 2019-2020)

Factors	Df	SS	MS	F
Barakatli 95				
A	1	22250.69	22250.69	150.455**
B	2	139253.76	69626.88	470.805**
C	2	17542.39	8771.19	59.309**

AB	2	355.85	177.92	1.203 ^{ns}
AC	2	334.06	167.03	1.129 ^{ns}
BC	4	69.03	17.26	0.117 ^{ns}
ABC	4	122.53	30.63	0.207 ^{ns}
	3	123.36	41.12	
Residual dispersion	72	10648.00	147.89	
Gobustan				
A	1	36322.01	36322.01	157.15 ^{**}
B	2	222628.60	111314.30	481.61 ^{**}
C	2	29900.51	14950.257	64.68 ^{**}
AB	2	619.01	309.51	1.339 ^{ns}
AC	2	263.43	131.72	0.570 ^{ns}
BC	4	198.40	49.60	0.215 ^{ns}
ABC	4	124.74	31.18	0.135 ^{ns}
Repetition	3	134.30	44.77	
Residual dispersion	72	16641.50	231.13	
ns: non-significant				
**: Significant at the probability level of 0.01				
A- Predecessor; B-soil cultivation; C- Nutritional condition				

Table 2. According to Duncan's criterion, variance analysis on the effect of predecessor, soil cultivation and nutritional conditions to the number of winter-surviving plants belonging to winter wheat varieties

Variants	Average price	
	Barakatli 95	Gobustan
Traditional (plowing+discing+ leveling at a depth of 20-22 cm	226.62 C	303.62 C
by one time discing of soil with a heavy trowel at a depth of 8-10cm,	269.77 B	360.19 B
by 2 times discing of soil with a heavy trowel at a depth of 8-10cm,	302.56 A	399.42 A
Without fertilizer	252.25 C	335.90 C
N ₉₀ P ₆₀ K ₄₅	267.50 B	356.29 B
N ₆₀ P ₆₀ + 10 t manure	279.21 A	371.04 A
a. The size of the average price used = 48.00		
b. Alpha = 0.05		

The significance of the effect of predecessors, soil cultivation and nutritional conditions on the amount of plants surviving the winter was estimated by analyzing the variance using the SPSS 26 program, and the results are given in Table 1. As a result of the variance analysis, due to both varieties it was revealed that the effect of each factor is significant at the probability level of 0.01. According to the Duncan model of variance analysis, the winter hardiness of plants has been high at the variant of 2 times discing the soil with a heavy trowel at a depth of 10-12 cm after the pea predecessor and using N₆₀P₆₀ + 10 t of manure.

Thus, peas belong to the group of cereals and legumes and accumulate more nitrogen in the soil. So, it has great importance with meeting the nitrogen needs of plants. The most common of the organic fertilizers is manure. When adding manure into the soil instead of a certain part of the mineral fertilizers, it maintains moisture in the soil by improving the physical properties of the soil, and macro and microelements required by plants enter the soil together with manure. As a result, the root system of plants

develops better and the plant is fully supplied with nutrients, leading to an increase in the winter hardiness of the plant. Also, in the South Mugan region, due to high summer temperatures and very low amount of rainfall, the soil remains too dry after the harvest of predecessors (winter wheat and peas) and deep plowing results in the formation of lumps of soil that aren't broken down into parts during pre-sowing cultivation and hinders the quality of sowing. When the soil is plowed twice with a heavy disk harrow at a depth of 10-12 cm, large lumps hindering the sowing aren't formed, and mass sprouts in the same size are obtained.

Conclusion

Thus, the results of our two-year study show that the winter wheat varieties under study are different in terms of winter hardiness considering their predecessors, soil cultivation and nutritional conditions. Thus, this indicator was higher in "Gobustan" wheat variety than in "Barakatli 95" wheat variety. However, relatively good performance for each variety was observed in post-pea tillage. The best results in soil cultivation were obtained in the variant of 2 times discing the soil with a heavy disk harrow at a depth of 10-12 cm. The highest winter hardiness for each variety was observed in the condition of twice disking of soil with a heavy disc trowel at a depth of 10-12 cm and by using $N_{60}P_{60} + 10$ t of manure.

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The Determination of the Optimum Parameters for the Formation of Silver Nanoparticles by *Aspergillus Niger* BDU-A4

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Abstract

In this work, the formation of silver nanoparticles was investigated by *Aspergillus niger* BDU-A4 at different stages/phases/culture media: a) pH: 4.82,5.79,7.42,8.88, 9.86;b) indicators of temperature (20°C, 25°C, 30°C, 37°C və 45°C) and c) indicators of different concentration of the salt of AgNO₃. As a result of UV-V, absorbance spectrum is defined as the optimal conditions for the formation of maximum amount of Ag nanoparticles from AgNO₃ - substrate by *Aspergillus niger* BDU-A4. This condition determined pH = 7.4 at 37 °C,with 10 grams of biomass within 1mm AgNO₃ solution in 100 ml suspension during 6 hours incubation.

Keywords: *Aspergillus niger*, Ag nanoparticles, pH, biomass, temperature, incubation period

Introduction

The size, shape, method of synthesis and other major properties of nanoparticles play an important role in the determination of practical importance (Mansoori *et al.*, 2007; Taylor *et al.*, 2013; Pundir, 2015).

The synthesis of metal nanoparticles as well as copper, zinc, titanium, magnesium, gold, cadmium and silver has particular importance among the synthesized nanoparticles. So they are widely used in the medical fields, manufacturing optical and electronic equipment, chemical industries, also agriculture in the form of elements and compounds (Mukherjee *et al.*, 2001a, 2001b; Bell *et al.*, 2003; Gilbert *et al.*, 2003; Chovanec *et al.*, 2005; Begum *et al.*, 2009; Rai & Ingle, 2012). The environmental risks of nanoparticles were investigated also by researchers as well as their synthesis and application (Fabrega *et al.*, 2014).

The optimal conditions were determined by different researchers for the synthesis of individual metal nanoparticles in different metal oxides and salts. Also the influence spectrum of various physical and chemical parameters was well investigated that affects the process of the synthesis of nanoparticles (Ottow & Von Klopotek, 1969; Sastry *et al.*, 2003; Vasilenko *et al.*, 2010; Desai *et al.*, 2011; Singh & Balaji Raja, 2011; Wu *et al.*, 2012; Li *et al.*, 2013; Ali *et al.*, 2014; Das *et al.*, 2014; Parmar *et al.*, 2014; Kameyama & Senna, 2014; Selishchev & Pavlishchuk, 2014; Yongju *et al.*, 2014; Aliakbari *et al.*, 2015; Makhdoomi *et al.*, 2015).

The antibacterial and inhibitor effects of silver along with its metal properties make it important to perform research on more effective ways to synthesize the silver (Jaidev & Narasimha, 2010; Fayaz *et al.*, 2012). Although the synthesis of silver nanoparticles is available with biological and other methods, "green" synthesis is more effective both financially and environmentally. The main cost - effective method in synthesis of nanoparticles is using microorganisms at "Green nanotechnology". Different species of bacteria and fungi that are products of nanoparticles haven't been investigated yet. Fungi synthesize silver nanoparticles from silver ions of their salts through intracellular and extracellular enzymes (Mukherjee *et al.*, 2001a; Ingle *et al.*, 2008; Castro-Longoria *et al.*, 2011; Jain *et al.*, 2011; Vahabi *et al.*, 2011; Abd El-Aziz *et al.*, 2012; Li *et al.*, 2012).

To increase the synthesis of silver nanoparticles with minimum investment by microorganisms, it is required to select the optimum culture conditions and suitable physical parameters for their products. Each of the selected active strains is able to be synthesized in different amount and form of silver nanoparticles/ synthesize different amount and form of silver nanoparticles, depending on the factors as follows: definition of biomass, pH, temperature, light intensity, nutritious environment, concentration, incubation period and so on. (Safekordi *et al.*, 2011; Yin *et al.*, 2012; Chan & Don, 2013; Sonal *et al.*, 2013).

It was determined that smaller - sized and spherical - shaped nanoparticles are synthesized at higher pH, while studying the correlation between the characteristics of silver nanoparticles and the environmental acidity. While at lower pH, relatively large, rod- or triangle-shaped nanoparticles are formed (Sonal *et al.*, 2013; Alqadi *et al.*, 2014). It was determined/found/revealed through the investigation of relation between the size of silver nanoparticles synthesized by fungi and temperature that the increasing of the reaction temperature leads to the reduction of nanoparticle size and their monodispersity (Fayaz *et al.*, 2009; Chan & Don, 2013; Sonal *et al.*, 2013).

The optimal concentrations of biomass and substrate were studied by researchers to increase the production of silver nanoparticles on an industrial scale by fungi and

bacteria (Korbekandi *et al.*, 2012; Chan & Don, 2013; Korbekandi *et al.*, 2013; Sonal *et al.*, 2013). The optimal physio-cultural conditions affecting the synthesis of silver nanoparticles was determined/measured/studied/found by Sonal and his collaborators. They have studied the effect of temperature and pH, biomass and substrate concentrations, as well as the effects of nutrient - rich environment, filtration level of suspension and intensity of light for optimal synthesis of nanoparticles by *Fusarium oxysporum* (Sonal *et al.*, 2013).

Plant extracts have been investigated as nanoparticle product and their optimal synthesis conditions was observed/recorded during the study of rapid synthesis of silver nanoparticles (Geetha *et al.*, 2013; Krishnaraj *et al.*, 2013; Thamer & Almashhedy, 2014; Christopher *et al.*, 2015; Pourmortazavi *et al.*, 2015).

At previous studies, the strains of mold fungus were separated from soil samples and different rotten plant residues to identify and test their ability to generate silver nanoparticles. It was studied that *Aspergillus niger* BDU-A4 active strain has the ability to form silver nanoparticles intensively (Ganbarov *et al.*, 2013; Ganbarov *et al.*, 2014a, 2014b). We also studied the relation of silver nanoparticles synthesis by *Aspergillus niger* BDU-A4 with the incubation period and the amount of biomass (Musayev *et al.*, 2013). The main purpose of the present study is to test other factors which can affect the formation of silver nanoparticles by *Aspergillus niger* BDU-A4 and identify the optimal performance of these factors for the rapid synthesis of silver nanoparticles.

Materials and Methods

Fungal culture: Weekly cultures of *Aspergillus niger* BDU-A4 taken from the cultures collection of Baku State University.

Reagents: For the synthesis of silver nanoparticles as the primary source, we used/applied 1mM AgNO₃ solution, also the solution of 1 N HCl and the solution of 1 M NaOH for preparation of solutions at relevant pH, and distilled water for preparation of solutions. The composition of the nutrient medium (g / l) was: - Sucrose-20; NaNO₃-3; K₂HPO₄-1; MgSO₄·7H₂O - 0.5; KCl- 0.5; FeSO₄·7H₂O-0.01.

UV-Vis spectral analysis of silver nanoparticles: For UV(UV-Vis) spectrophotometer, "Analytic Jena" spectrophotometer was used/applied (model: Specord - 250 pulse, German). UV spectrophotometer (UV-vis) is a valuable tool for determination, characterization and study of nanomaterials. UV-vis is the equipment which calculates the dispersed or absorbed light of a given sample at the solution. The suspension of silver nanoparticles has dark golden-yellow color through the

surface plasmonic resonance. These are the motions of electrical conductance as a response to electromagnetic waves. Silver nanoparticles have a spectrum absorption characteristic at the "vision" circle of UV-vis. Spectral characterization of silver nanoparticles strongly depends on their size, shape, inter-particle gaps and surroundings (Rao *et al.*, 2002). The dependency of quantum size affects the passage of red or blue spectra of surface plasmonic resonance. Therefore, the absorption peaks can be used as a means in determining particles' size and stability. Nano-scaled silver nanoparticles form peak at the wavelength of 400 - 420 nm. However, when the size of particle increases, its absorption spectrum goes beyond nanoscale.

Synthesis of silver nanoparticles: The fungal culture was incubated in 250 ml Erlenmeyer flasks that contain 100 ml nutrient medium, at 28°C, at 120 rpm, within 120 hours. Received biomass washed by distilled water, it was incubated again within 24 hours with 100 ml of distilled water at 28 °C, to clean from nutrient medium completely. Then the suspension is drained through Whatman filter paper (№1) and the gained/collected wet biomass is weighted. For the synthesis of silver nanoparticles, 100 ml of suspension was prepared that consisted of 10 grams of biomass, 1 ml of distilled water and 1 mM AgNO₃ in 250 ml Erlenmeyer flasks. Suspensions were incubated at different pH and temperature in dark conditions. Silver nanoparticles were synthesized as a result of reduction of silver ions to metallic silver at the optimal indicator of each factor after incubation.

Selection of the optimal conditions for the synthesis of Ag nanoparticles:

In order to ensure fast and stable synthesis of silver nanoparticles by *Aspergillus niger* BDU-A4, we studied the effects of following factors: medium acidity (pH), temperature, incubation period, the amount of biomass and concentration of silver nitrate. The experiments were carried out twice throughout the study of each physical and cultural indicator.

a) Effect of pH on the formation of nanoparticles

The biomass was incubated in solutions with a pH of 4.8, 5.8, 7.4, 8.9 and 9.9 to study the effect of pH on the formation of nanoparticles.

b) Effect of temperature on the formation of nanoparticles:

The suspension was incubated in a thermostat at stable temperatures of 20 °C, 25 °C, 30 °C, 37 °C and 45 °C to study the effect of temperature on the formation of nanoparticles (**experimental procedure is shown above**).

c) Effect of concentration of AgNO_3 salt on the synthesis of nanoparticles:

10 grams of biomass was incubated in AgNO_3 salt solutions in different concentrations of 0.5 mM, 1 mM, 2 mM, 3 mM and 5 mM to study the effect of concentration of AgNO_3 salt on the synthesis of silver nanoparticles.

Results and Discussion

a) Effect of pH on the formation of nanoparticles

The formation of silver nanoparticles by *Aspergillus niger* BDU-A4 was investigated at different pH values (4.8, 5.8, 7.4, 8.9, 9.9) in UV-vis spectrophotometer and the results obtained are shown in Figure 1. According to the results of spectrum, the synthesis of silver nanoparticles has been observed at all tested pH values.

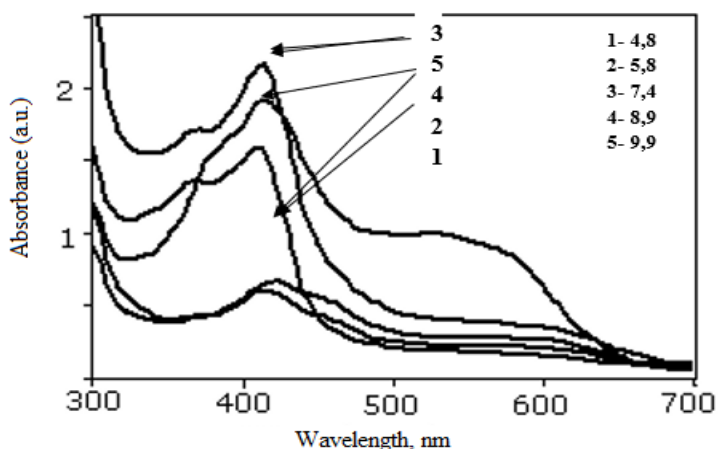


Figure 1. The effect of pH on the formation of silver nanoparticles by *Aspergillus niger* BDU-A4

But it is known that the maximum synthesis of silver nanoparticles occurs in an alkaline environment (8.9 and 9.9pH values). According to reduction of pH value from pH 7.4 toward acidic environment, the amount of synthesized nanoparticles is decreased, and when the pH level reaches 4.8, it results in aggregation of particles. After incubation, the flocculation of particles was not observed in none of the solutions with different pH values. However, after one week the aggregation of particles occurred in a solution of 4.8 pH. But in solutions of alkaline, stable diffusion of the nanoparticles was observed.

Stable diffusion or aggregation of silver nanoparticles in solutions may depend on zeta potential of particles and the amount of OH⁻ anions in solution. Thus, the zeta potential of particles is high in alkaline environment as a result of adsorption of OH⁻ anions by the silver nanoparticles. Thereby, the electrostatic repulsion force formed in particles provides their sustainability in solution. Lack of anions OH⁻ weakens zeta potential of particles in the acidic environment that makes their aggregation inevitable (Sonal *et al.*, 2013).

b) Effect of temperature on the formation of nanoparticles:

The formation of silver nanoparticles by *Aspergillus niger* BDU-A4 was studied at different temperatures of 20 °C, 25 °C, 30 °C, 37 °C, 45 °C in UV-vis spectrophotometer and the results are shown in Figure 2. According to analysis of spectra, the synthesis percentage and surface plasmonic absorption of silver nanoparticles increase gradually as the temperature increases.

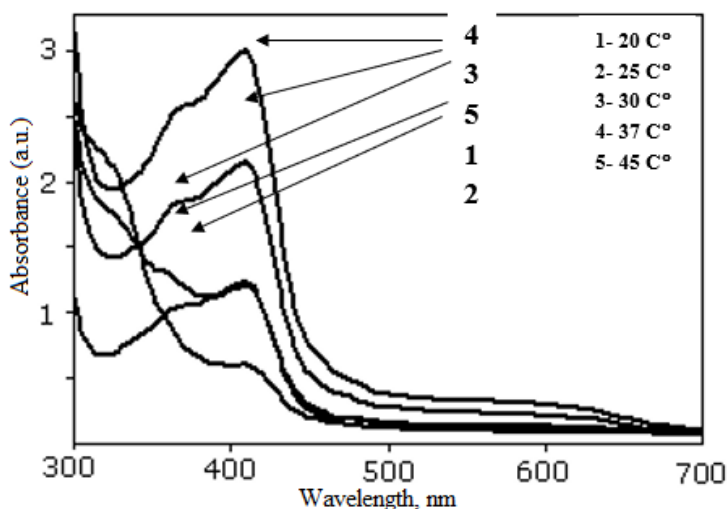


Figure 2. The effect of temperature on the formation of silver nanoparticles by *Aspergillus niger* BDU-A4

The highest density of nanoparticles is observed at 37 °C but reduced at 45 °C respectively. Such a change in absorption and the increase in surface plasmonic resonance report an increase in the number and size of silver nanoparticles in the suspension of flasks and directly cause the solution to get dark silver color.

c) Effect of concentration of AgNO_3 salt on the synthesis of nanoparticles:

10 grams of biomass was incubated in AgNO_3 salt solutions in different concentrations of 0.5 mM, 1 mM, 2 mM, 3 mM and 5 mM to study the effect of concentration of AgNO_3 salt on the synthesis of silver nanoparticles and the formation of nanoparticles was tested in UV-vis spectrophotometer. The obtained results are shown in Figure 3.

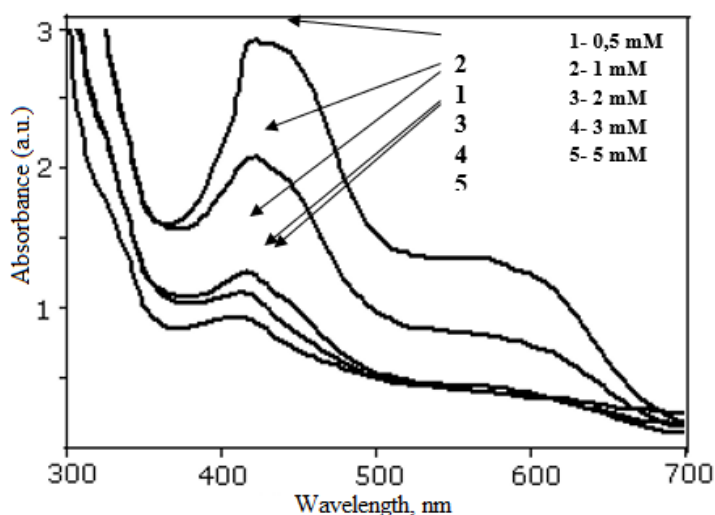


Figure 3. The effect of different concentrations of AgNO_3 salt on the formation of silver nanoparticles by *Aspergillus niger* BDU-A4

According to analyses of spectra, the surface plasmonic adsorption was also increased as the concentration of silver-nitrate increased. But the highest density of silver nanoparticles was observed in 1 mM concentration of silver nitrate.

Conclusion

In this research, we have studied the effect of certain parameters of physical conditions in synthesis of silver nanoparticles: medium acidity (pH), the effect of temperature and the concentration of AgNO_3 salt by active strains of *Aspergillus niger* BDU-A4. The effect of different pH values was analyzed in UV-Vis spectroscopy and it was revealed that the optimum pH value for the highest synthesis

of silver nanoparticles is 7.4 at 28 °C in suspension of 1mm AgNO₃ and 10 grams of biomass. According to UV-Vis analysis of temperature effects, the optimum temperature is 37 °C for getting the highest density of silver nanoparticles in the suspension of 1mm AgNO₃ and 10 grams of biomass. Also, it was determined/observed/revealed that more synthesis of Ag nanoparticles is observed in the solution of 1mm concentration of AgNO₃ salt.

Finally, optimization of the medium acidity (pH), effect of temperature and concentration of salt AgNO₃, which affect the synthesis of silver nanoparticles by *Aspergillus niger* BDU-A4, can be used in the synthesis of silver nanoparticles on an industrial scale and in the production of substances with antimicrobial properties.

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Approaches to Breast Cancer Diagnosis

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Abstract

Breast cancer is the most common cancer in women, accounting for about one-third of cancer cases in women and approximately 14% of cancer-related death in females in the worldwide. It is of great importance to detect breast cancer in the early stage and avoiding overtreatment in patients who only receive a modest benefit, while suffering from toxic side effects, so aiming early detection, diagnostic strategies are important. Biomarkers that are found in blood, urine or body tissues are mostly useful in evaluating the progression of the disease status after initial chemotherapy and radiotherapy to monitor subsequent treatment strategies. In this review, we discuss the importance of established prognostic factors and predictive biomarkers as well as some emerging biomarkers that are currently undergoing testing for technical validity and clinical utility.

Keywords: Breast cancer, Biomarkers, diagnosis, prognosis,

Introduction

Breast cancer is the most common cancer in women, accounting for about one-third of cancer cases in women and approximately 14% of cancer-related death in females in the worldwide (Duffy et al., 2015). Breast cancer includes a heterogeneous group of tumors with a wide spectrum of morphologically and molecularly different subtypes which display different risk factors, clinical and histopathological features and response to systemic treatments (Dai et al., 2016, Duffy et al., 2015). Therefore, It is of great importance to detect breast cancer in the early stage and avoiding overtreatment in patients who only receive a modest benefit, while suffering from toxic side effects (Dai et al., 2016, Weigel and Dowsett, 2010).

Biomarkers that are found in blood, urine or body tissues are mostly useful in evaluating the progression of the disease status after initial chemotherapy and radiotherapy to monitor subsequent treatment strategies (Kabel, 2017). Based on the clinical role of biomarkers, they are including prognostic, predictive and

pharmacodynamics biomarkers. Gene expression signatures that are related to the risk for recurrence and tumor stage can be considered prognostic biomarkers in breast cancer. While prognostic markers do not predict whether a particular therapy will be successful or not predictive biomarkers including, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) associated with the optimal therapies for patient care. Pharmacodynamics biomarkers measure the proximal effect of a drug on its target. The demonstration of declined phosphorylation of a protein substrate immediately downstream from a target kinase is example of proximal pharmacodynamics effect. Peripheral blood mononuclear cells and skin that are easy-access tissues have used for measurement of pharmacodynamics biomarkers.

Therefore, biomarker analysis in breast cancer not only provides additional information about classical clinical factors, but also enables patients with a more favorable benefit-risk balance to receive certain treatments (Colomer et al., 2018, Ern Ang et al., 2012, Ulaner et al., 2016). In this review, we discuss the importance of established prognostic factors and predictive biomarkers as well as some emerging biomarkers that are currently undergoing testing for technical validity and clinical utility.

1- Biomarkers and Breast cancer

The term “biomarker” refers to a broad subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly. The WHO has defined that biomarkers include “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction. Examples of biomarkers include everything from pulse and blood pressure through basic chemistries to more complex laboratory tests of blood and other tissues (Organization, 1993, Strimbu and Tavel, 2010). Biomarkers are often protein markers, such as prostate-specific antigen for the detection of prostate cancer, and genomic markers, such as epidermal growth factor receptor (EGFR) kinase mutations in non–small cell lung cancer, which predict response to EGFR kinase inhibitors (Ulaner et al., 2016). Biomarkers currently play an indispensable role in the management of patients with breast cancer, especially in deciding the type of systemic therapy to be administered (Duffy et al., 2017). Among the molecular markers associated with breast cancer, the estrogen receptor (ER), the progesterone receptor (PR), the human epidermal growth factor receptor (HER2) and

the Mib1/Ki-67 proliferation index are the most important ones and are firmly established in the standard care of all primary, recurrent, and metastatic breast cancer patients (Beenken and Bland, 2002).

2-1 Imaging and Emission-Based Systems

2-1-1 PET scan

A positron emission tomography (PET) scan is an imaging test that uses a radioactive substance (called a tracer) to look for potential spread of breast cancer. This tracer can help identify areas of cancer that an MRI or CT scan may not show. PET has the ability to demonstrate abnormal metabolic activity, and 18F-2-deoxy-D-glucose (FDG) PET provides important tumor-related qualitative and quantitative metabolic information that may be critical for the diagnosis and follow-up (Kubota et al., 1989, Minn and Soini, 1989, Wahl et al., 1991). The ability of PET to detect breast cancer depends on the tumor's size and histology. The sensitivity of PET has been reported to be 68% for small (< 2 cm) tumors and 92% for larger (2-5 cm) tumors (Avril et al., 2000), and its reported overall accuracy for detecting in situ carcinomas is low (sensitivity: 2-25%). The major limitation of PET or PET/CT for breast imaging is its poor detection rate for small breast carcinomas and non-invasive breast cancers (Noh et al., 1999, Schirrmeister et al., 2001).

2-1-2 Computed Tomography (CT scan)

Computed tomography (CT) imaging based on the variable absorption of x rays by different tissues, also known as "CAT scanning" (Computerized Axial Tomography), provides a different form of imaging known as cross-sectional imaging, this types of images (Figure 2) are used for a variety of diagnostic and therapeutic purposes (Administration, 2020). CT imaging is reliable for Breast cancer diagnosis because it can disclose every suspected and unsuspected cancer nodules (Gindi et al., 2014). However, variance of intensity in CT scan images and anatomical structure misjudgment by doctors and radiologists might cause difficulty in marking the cancerous cell (Suzuki et al., 2006). Imaging modalities like CT rely on detecting anatomic changes for the diagnosis, staging and follow-up of cancer patients (Yang et al., 2007).

2-1-3 Magnetic Resonance Imaging (MRI)

The basis of magnetic resonance (MR) techniques is the measurement of radiofrequency radiation resulting from transitions induced between nuclear spin

states of tissue hydrogen atoms (protons) in the presence of a strong external magnetic field (Khoo et al., 1997).

MRI is used widely both for screening women who are at increased risk of breast cancer and for treatment selection. MRI may prove useful in screening younger women with dense breasts who are at a special high risk of developing breast cancer (Morrow et al., 2011). MRI has a higher sensitivity for the detection of breast cancer and is not affected by breast density (Sardanelli et al., 2004). Regarding all mentioned above MRI precision also relies on detecting anatomic changes for the diagnosis, staging and follow-up of cancer patients (Yang et al., 2007).

2-1-4 Mammography

Screening with mammography has the ability to detect breast cancer at an early stage. Subsequent effective diagnostic pathways and treatment regimens can reduce the burden of disease of breast cancer, most importantly mortality in women aged 50 to 70 years (US, 2009). Randomized trials have shown that mammographic screening of all women who are between 50 and 70 years of age can reduce mortality from breast cancer by about 25 percent (Kriege et al., 2004). In women with breast cancer, disease burden is the main determinant of the selection of local therapy, and women selected for breast conserving surgery with mammography successfully complete the procedure in more than 85% of cases. (Morrow et al., 2011). Although the benefits of mammography are proven, not all cancers can be visualized on screening mammograms. The sensitivity of mammography is related to the age, ethnicity, personal history, and technique quality. The sensitivity of mammography is decreased in women with dense breast tissue, and some women who seem to have localized cancer mammographically are found to have extensive disease necessitating mastectomy (Wang, 2017).

2-2 Elisa-based Markers

2-2-1 CA 15-3

Along with the traditional pathological factors and molecular markers, serum tumor markers have an important role in monitoring therapy, early diagnosis of recurrence, determining prognosis, and treatment of many malignancies. The most widely used serum markers in breast cancer are carcinoembryonic antigen (CEA) and cancer antigen 15–3 (CA15-3) (Shao et al., 2015).

CA 15-3 peptides are shed or soluble forms of MUC- 1, which exists as a transmembrane protein consisting of two subunits that form a stable dimer (Kabel,

2017). CA 15-3 in combination with CEA is the most widely used serum marker in patients with breast cancer (Cristofanilli et al., 2005). Accordingly, the European Group on Tumor Markers has mentioned the CEA and CA15-3 levels can be used for assessing prognosis, the early detection of disease progression, and treatment monitoring in breast cancer (Molina et al., 2005). Using CA 15-3 has its disadvantages, CA 15-3 levels may also be increased in several benign and malignant conditions. This results in low sensitivity, specificity, and positive predictive values, making it difficult to reliably screen, diagnose, or stage breast cancers. CA 15-3 is only elevated in 10% of patients with early-stage breast cancer, and levels of CA 15-3 can also be elevated due to lung and ovarian cancers. Furthermore, the polymorphic, glycoprotein structure of MUC1, detected by CA 15-3 and CA 27.29, presents similar assay problems to those described for other mucins such as CA 19-9 (Wild, 2013).

2-2-2 CA 27.29

Cancer antigen (CA) 27.29 is a monoclonal antibody to a glycoprotein (MUC1) that is present on the apical surface of normal epithelial cells (Gion et al., 1999). The molecule exists in a normal, highly glycosylated form, and a cancer-associated, relatively underglycosylated form (Beveridge, 1999). CA 27.29 is highly associated with breast cancer. However, this mucin is not specific to breast cancer and is considered a pan-epithelial marker. It is also expressed on other adenocarcinomas, including lung, colon, pancreas, and ovary (Hayes et al., 1985). The CA 27.29 level is elevated in approximately one third of women with stage I or II breast cancer and in two thirds of women with late-stage disease (Perkins et al., 2003). Due to the current tumor marker guidelines of the American Society of Clinical Oncology, CA 15-3 and CA 27.29 are not recommended as prognostic markers for routine clinical use because there are no trials available demonstrating a clear benefit regarding improved survival or diminished toxicity resulting from a timely detection of recurrence and early treatment initiation (Rack et al., 2010). Despite these drawbacks, testing for the existence of tumor markers is widely used in disease surveillance and treatment monitoring in daily practice. As non-invasive, reproducible and easily accessible tests are available at any point in time during disease progression for CA 27.29 markers they are a highly suitable measure by which to select patients at risk of recurrence, both at primary diagnosis and during follow-up, and to monitor treatment efficacy (Laessig et al., 2007, Rack et al., 2010).

CA 27.29 has some drawback like CA 15.3, CA 27.29 is not elevated in all patients with breast cancer and also it could be elevated in some noncancerous and cancerous conditions other than breast cancer (Wild, 2013).

2-2-3 CEA

Carcinoembryonic antigen (CEA), an oncofetal glycoprotein and type of cell adhesion molecule, is expressed in normal mucosal cells and overexpressed confirmed (Perkins et al., 2003). CEA was one of the first tumor markers to be studied and characterized as prognostic factors in breast cancer for more than 30 years and the most common tumor markers used in breast cancer. Several studies have showed that an increase or a decrease in the CEA levels may reflect the status of disease progression or regression and correlate with the stage of disease (Guadagni et al., 2001). CEA levels in the blood are usually increased once the cancer has metastasized. However, CEA levels typically return to normal within four to six weeks after successful surgical resection (Wu et al., 2014). Recently, the prognostic value of preoperative CEA and CA15-3 levels in breast cancer has gained much attention. Plasma CEA levels combined with CA15-3 levels may provide useful information for diagnosis and treatment of breast cancer (Ebeling et al., 2002, Park et al., 2007).

2-3 Tumor-based Markers

2-3-1 Estrogen receptor (ER)

Estrogen receptors (ERs) belong to the subfamily of ligand-regulated transcription factors which transduce hormones signaling into a large variety of physiological responses in various organs. This steroid hormone receptor is one of the successful tumor markers in breast cancer which was first identified in the late 1960s. ER found on nearly 70% of primary breast cancers and plays an important role in tumor progression. ER exists in two main forms, ER α and ER β . Whereas the original ligand-binding ER assays are likely to have detected both of ER forms, the current immunohistochemistry (IHC) measurements detect only ER α (Duffy et al., 2017, Kabel, 2017). However, for both ER α -positive breast cancer and ER α -negative one, ER β can be observed, which plays a key role in breast cancer classification and endocrine therapy (Osborne, 1998). ER α is responsible for estrogen-induced mitogenic signaling in epithelial cells in breast, uterine, and ovarian tissues and is prevalently expressed by breast cancer cells, whereas ER β is usually associated with less aggressive tumors, as it inhibits both ER α -mediated transcription and estradiol-induced proliferation in various types of cancer cells. The ER α /ER β ratio may play a critical role in the regulation of estradiol activity in breast cancer cells (Matthews and Gustafsson, 2003, Paruthiyil et al., 2004). Five lysines on ER α are reportedly acetylated by p300: Lys266, Lys268, Lys299, Lys302 and Lys303, all localized in the hinge region. The effects of ER α acetylation result from a two-step mechanism:

short exposure of cells to HDAC inhibitor (HDACi) leads to acetylation and stabilization of the receptor, whereas after long exposures, the receptor is delocalized and subsequently degraded by the proteasome (Paruthiyil et al., 2004). While the absence or presence of the ER is used to obtain treatment decisions, little attention has been paid on the value of the quantitative expression levels as a predictive indicator. The Early Breast Cancer Trialists' Collaborative Group reported that higher levels of ER were associated with a lower risk of recurrence when receiving adjuvant tamoxifen (Weigel and Dowsett, 2010). Tamoxifen is a selective ER modulator (SERM) and the most frequently used anti-estrogen adjuvant treatment for ER+ pre-menopausal women. Tamoxifen exhibits antagonistic effects in breast tissue, thus has preventive effects on breast cancer development and cytotoxic effects on breast cancer cells (Egeland et al., 2015). The ER has a role in cellular growth, proliferation and differentiation. When ER interacts with estrogen, they will regulate transcription of specific genes, such as PR, TFF1, GREB1 and PDZK (Ern Ang et al., 2012). In addition to prognostic value, ER is the most important biologic marker of response to treatment in breast cancer. Clinically, ER status is a critical index of sensitivity to endocrine therapies because ER-positive breast cancers use estradiol as a main growth stimulus. While endocrine therapy with 5-year tamoxifen as an adjuvant decline the ER-positive breast cancer death rate, ER-negative disease illustrates no significant benefit from this treatment except in the unusual type of tumor; ER-negative but PR-positive tumors (Group, 2005). Multiple clinical studies have demonstrated that the ER-negative breast cancer patients are more likely to achieve a pathological complete response (pCR) with neoadjuvant chemotherapy than the ER-positive patients, with pCR rates of 7–8 vs 21–33% respectively (Colleoni et al., 2004).

2-3-2 Progesterone Receptor (PR)

The progesterone receptor (PR) is a well-known estrogen receptor (ER)-regulated gene that is expressed in over two-thirds of ER-positive breast cancers. Like ER, PR protein exists as two receptor isoforms namely A and B, but these forms are the products of the same gene. These isoforms of PR (A is a slightly truncated form of B) bind with one another to create homo- and heterodimers (Hammond et al., 2010). The potential of PR expression as a prognostic biomarker has been appreciated since 1975 when it was first suggested that PR expression could predict outcome and response to ER-directed therapy in advanced disease. PR is more highly expressed in the luminal A breast cancer subtype, and is associated with tumor grade, ER expression as well as negative HER2 status in early breast cancer (Lim et al., 2016, Purdie et al., 2014). In positive ER breast cancers, PR is often used as a positive prognostic marker of disease outcome. There is increasing evidence that substantial crosstalk occurs between ER and PR signaling pathways. Noticeably, when PR is

activated by its native ligand in the presence of estrogen, it interacts with ER in breast cancer cells to redirect ER chromatin binding, signifying the critical role PR plays in modulating ER action (Mohammed et al., 2015). Additionally, high levels of PR associated with decreased metastatic events in early stage disease and administration of a progesterone injection prior to surgery can provide improved clinical benefit (Bardou et al., 2003, PichÃ³n et al., 1980).

2-3-3 HER2

Human epidermal growth factor receptor 2 (HER2) is a transmembrane member of the tyrosine kinase epidermal growth factor receptors, which are normally expressed at low levels in all epithelial cells in normal fetal and adult tissues, but are also essential for cancer proliferation and survival. HER2 gene amplification has been associated with increased levels of expression of HER2 mRNA and protein product, which lead to oncogenic signaling and resultant self-sufficiency in growth signals, uncontrolled proliferation, sustained angiogenesis, enhanced invasion, and metastasis processes, which are drivers of tumor development and progression in a subset of breast cancer (Beenken and Bland, 2002, Schwarzenbach et al., 2012). HER2 enriched breast cancer reports between 20% and 30% of all breast cancer. It is characterized by over expression of *HER2/neu* proliferation genes with low expression of luminal clusters including CK7, CK8, CK18, CK19 and other luminal-associated markers like X-box-binding protein 1, hepatocyte nuclear factor 3, GATA-binding protein 3 and estrogen receptor 1, among other (Kittaneh et al., 2013). Several potential clinical applications have been suggested for determination of HER2 status in breast cancer patients such as determination of prognosis in untreated patients, prediction of resistance to endocrine therapy or of selective resistance to tamoxifen but not aromatase inhibitors, prediction of relative resistance to certain chemotherapies like cyclophosphamide, methotrexate, and fluorouracil (CMF) –like regimens and prediction of benefit from anti-HER2 therapies, in particular trastuzumab (Harris et al., 2007).

Trastuzumab, the most well-known humanized monoclonal antibody against HER2, dramatically improves response rates, time to progression and survival when used both alone and/or with chemotherapy in both early stage and metastatic breast cancer. Other HER2-targeted drugs, including lapatinib as a tyrosine kinase inhibitor, the antibody pertuzumab, and the antibody drug conjugate adotrastuzumab emtansine (T-DM1), improve outcomes in HER2-positive metastatic breast cancer (Geyer et al., 2006, Slamon et al., 2011).

HER2 gene amplification is directly associated with its mRNA expression and protein levels, therefore, overexpression of the HER2 protein product may be

evaluated by Western blotting, ELISA or IHC; overexpression of its mRNA by Northern blotting or RT-PCR, and its gene amplification by fluorescence (FISH), chromogenic (CISH) or silver-enhanced in situ hybridization (SISH) (Hammond et al., 2010). Among of all, IHC has been more widely used as the primary test for HER2 status due to its results quicker, permits parallel viewing of tumor morphological features, and stained tissues do not degrade over time (Penault-Llorca et al., 2009).

2-3-4 Ki-67

Ki67 is associated with biologic breast cancer markers which may have a role in clinical practice as prognostic, predictive factors and possible targets for future therapies (Wiesner et al., 2009). The Ki-67 antigen, a non-histone protein was originally identified by Gerdes and colleagues in the early 1980s, by use of a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma-derived cell line (Yerushalmi et al., 2010). The Ki-67 antigen can be identified by immunostaining with a monoclonal antibody in all phases of cell proliferation. Non-existent in the resting (G0) phase, it appears within the nucleus in the S, G1 and G2 phases (Mannell, 2016). Ki-67 score is the most often measured on histological sections by IHC methodology and is defined as the percentage of stained invasive carcinoma cells (Kabel, 2017). It is really characteristic that Ki67 is expressed exclusively in estrogen receptor (ER)-negative cells, which means that ER-positive cells do not proliferate under normal circumstances. This separation does not exist in malignant tissues (Kontzoglou et al., 2013).

Many studies have shown that Ki67 can be used as a prognostic marker for breast cancer.

The study by Cheang and colleagues describes an immunopanel of ER, PgR, HER2, and Ki67 that can segregate the luminal A and B subtypes in a similar manner to that defined by a 50-gene expression profile. Luminal breast cancers with Ki67 levels of at least 14% were assigned to the luminal B category and had a worse prognosis for both breast cancer recurrence and death compared with luminal A subtype with Ki67 levels of less than 14% (Cheang et al., 2009, Yerushalmi et al., 2010).

Apart from the contribution of Ki-67 to prognosis, the Ki-67 index is used on a daily basis in the selection of therapy. Dividing cells have increased sensitivity to cytotoxic drugs, and a high Ki-67 is associated with a good response to neoadjuvant chemotherapy (NAC) (Fasching et al., 2011, Mannell, 2016). The prognostic and predictive value of Ki-67 was evaluated by Luporsi et al. and they concluded that this biomarker could be considered as a prognostic factor for therapeutic decision;

however, standardization of techniques and scoring methods are needed for integration of this biomarker in everyday practice (Luporsi et al., 2012).

2-4 Molecular markers

2-4-1 MicroRNAs

MicroRNAs (miRNAs) are a 21–25 long class of small non-coding RNA, which are capable of altering gene expression post-transcriptionally by inhibiting translation of their target mRNAs. miRNAs have been demonstrated to be involved in cell development, differentiation, proliferation and apoptosis (McGuire et al., 2015). miRNA can exert their action in cancers through both tumour suppression and oncomirs (by oncogenic mechanisms). The first human, disease-related miRNA characterized was from chronic lymphocytic leukaemia and subsequently, circulating miRNA were identified in patients with diffuse large B-cell lymphoma (Shi et al., 2010, Wang and Wang, 2012). The potential of miRNA as biomarker targets is facilitated by their stability in blood as well as formalin-fixed paraffin-embedded tissues and their ability to withstand repeated freezing and thawing cycles (Mitchell et al., 2008). Significantly, as miRNAs have been implicated in cancer metastasis, miRNA signatures are being pursued as novel clinical diagnostic targets to allow further subtyping of breast cancer and for predicting metastasis or therapeutic resistance (Shi et al., 2010). Studies have showed that miRNAs link to all stage along the metastatic cascade in breast cancer (Jang et al., 2014). The first miRNA shown to be highly expressed in metastatic breast cancer was miR-10b, with a clinical correlation in primary breast carcinomas. Surprisingly, a noticeable increase in circulating miR-10b and miR-373 was illustrated in lymph node positive patients, in comparison to patients with no nodal involvement or healthy controls. Admittedly, miR-21 has also been identified as a marker for breast cancer and predictor of stage (Asaga et al., 2011, Ma et al., 2007, Schwarzenbach et al., 2012). MiR-155, the most widely studied circulating miRNA in breast cancer, has been associated with ER/PR/HER2 expression. It is upregulated in the serum of breast cancer patients compared to healthy controls. The expression levels of miR-155 decreases significantly in metastatic breast cancers compared to primary cancer and negative control. As a result of these findings, it has been classed as a stable biomarker for breast cancer, confirmed by a meta-analysis of circulating miRNAs in breast cancer (Mathe et al., 2015, Roth et al., 2010, Wang et al., 2010). Recent studies have investigated that microRNAs namely, miR-210, miR-328, miR-484 and miR-874 have the potential to predict prognosis or risk of breast cancer recurrence (Volinia and Croce, 2013). Additionally, many findings have sowed that miRNAs

play a key role in regulating the sensibility of breast cancer cells to chemotherapy. miR-218 regulates cisplatin chemosensitivity by targeting BRCA1 as well as miR-451 and miR-326 were found to down-regulate the expression of MDR-1 and MRP-1, respectively, resulted in increased sensitivity of MCF-7 cells to doxorubicin. Some miRNAs can cause breast cancer cells to grow without estrogen and develop resistance to endocrine therapy by down-regulating the expression of ER α (Ji et al., 2019). Identifying circulating miRNA to use as biomarkers for metastatic breast cancer is presently a key priority for many research groups. A noticeable increase in circulating miR-10b and miR-373 was presented in lymph node positive patients, compared to patients with no nodal involvement (Mitchell et al., 2008).

2-4-2-cfDNA

The presence of circulating, cell-free nucleic acids in the bloodstream was first described by Mandel and Métais in 1948 (Leon et al., 1977). Circulating tumor cells (CTCs) and circulating nucleic acids such as cell-free DNA (cfDNA) have a potential to use for cancer screening, prognosis determination, and monitoring of the efficacy of anticancer therapies (Chimonidou et al., 2013). cfDNAs are considered a promising new diagnostic tool, especially for patients with advanced-stage cancer, in whom the cfDNAs can be used as a “liquid biopsy,” allowing physicians to follow cancer changes over time and tailor treatment accordingly (Pantel et al., 2009). The size of cfDNA may indicate its source. For example, apoptotic cells release DNA fragments of 180–200 base pairs whereas higher molecular-weight DNA fragments of over 10,000 bp in size are produced by necrotic cells (Jahr et al., 2001).

The release of cfDNA into the bloodstream occurs by different sources, including the primary tumor, tumor cells that circulate in peripheral blood, metastatic deposits present at distant sites, and normal cell types. Thus, both tumor and normal cfDNA circulate in the bloodstream of patients with cancer (Schwarzenbach and Pantel, 2015). Genetic alterations found in blood from patients with breast cancer include mutations, loss of heterozygosity, and altered methylation patterns. These alterations detected in the primary tumor may also be found in plasma/serum cfDNA of patients with Breast cancer (Li et al., 2012). The detection of tumor-specific DNA alterations in cfDNA provides a less invasive, more easily accessible source of DNA for genetic analysis than tumor biopsies (Skvortsova et al., 2006). Using fluorometry, showed the continuous increase in plasma cfDNA during tumor progression and its decrease after surgery (Tangvarasittichai et al., 2015).

The tumor-derived fraction of this total cfDNA, is under wide investigation as diagnostic and prognostic biomarker in several types of cancer, including breast,

lung and colon cancers(Fernandez-Garcia et al., 2019). This studies are using genomic alterations, such as methylations or tumor-specific mutations, the most valuable factors that allow us to precisely distinguish circulating DNA from normal-cell and tumor circulating ctDNA(Warton et al., 2016). Methylation aberrations are frequent features of many malignant diseases and can be detected in serum/plasma ctDNA when released into the bloodstream. Changes of methylation status usually occur in an early stage of carcinogenesis and hence are considered to be a better diagnostic factor than DNA mutations(Baylin and Jones, 2011). The DNA methylation pattern is often consistent between the cfDNA and the DNA from its tissue origins in cancer. This fact opens many opportunities for applying DNA methylation changes to the field of cancer diagnosis. The use of methylation status as a biomarker for cancer detection has several advantages over the methods established on genetic differences(Gai and Sun, 2019). First, epigenetic alterations are similar between any two tumors of the same type (same tissue origin)(Kundaje et al., 2015). Second, a methylation profile is tissue specific as well as constant between several tissue types among different patients. Consequently, investigating plasma DNA allows one to specify the tumor origin of cfDNA(Gai and Sun, 2019).

Conclusion

This paper reviewed the current most commonly available screening and biomarkers for diagnosing early and late stage breast cancer. The standard and new techniques based on tumor and blood markers, imaging and emission-based systems and molecular markers in diagnosis approaches for breast cancer detection were reviewed. Based on the above, it is clear that biomarkers are currently playing an important role in the management of patients with diagnosed breast cancer. Among all reviewed markers, molecular markers like cfDNA and microRNA have been suggested as a remarkable breast cancer markers duo to the detection of tumor-specific DNA alterations such as mutations and methylation in cfDNA provides a less invasive, more easily accessible source of DNA for genetic analysis than tumor biopsies. In addition, detecting somatic mutations from plasma DNA in advanced cancer patients may be potentially preferable when repeated tumor biopsies are not feasible and genomic analysis of archival tumor is deemed insufficient. However, molecular markers are still not mature and many challenges need to be solved before they can be implemented for clinical trials. A further urgent need is the identification and validation of biomarkers for predicting response to specific forms of chemotherapy.

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Pathologoanatomic Changes in Birds with Associated Infections (Infectious Bronchitis and Escherichiosis)

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Abstract

Infectious diseases tend to be widespread in large poultry complexes containing a large number of birds in limited areas. It is an actual problem for industrial poultry farming. The presence of infectious diseases of viral and bacterial etiology in poultry farms has a negative effect not only on the exacerbation of the epizootic situation, but also it leads to a sharp decline of economic indicators. These infectious diseases are often associated and considered as a medical and environmental problem, due to the risk of spread among people. Experimental infection of chickens causes a slowdown in the growth and loss of cilia of epithelial cells, dilatation of tubular glands, infiltration of lymphocytes, mononuclear cells, plasma cells, as well as edema and fibroplasia of the proper layer of all parts of the oviduct. So, the clinical signs of IBC and its mixed course with escherichiosis, pathological changes and histological examination allow us to establish a preliminary diagnosis.

Introduction

Infectious bronchitis of chickens (IBC), the causative agent of which is the coronavirus, is one of the frequently recorded diseases in poultry complexes. The problem associated with this disease has become more actual and urgent because of the pandemic caused by this group of coronaviruses. Coronaviruses, the causative agents of human respiratory diseases, differ from the infectious bronchitis virus (IBV) in birds by the sequence of proteins in RNA and antigenicity (Cavanagh, 1995).

It has been established that there is a low titre of neutralizing antibodies against IBV (Brown et al., 1987; Cook & Huggins, 1986; Cowen et al., 1987) in the blood serum of people caring for chickens. This fact still requires further thorough investigation.

The high contagiousness of the disease and the multiplicity of serotypes of the causative agent of infectious bronchitis in chickens complicate significantly the

whole process of immunization and increase the cost of attempts to prevent this illness in this way.

IBC is a highly contagious respiratory disease characterized by symptoms of respiratory tract and nervous system damage, nephrotic, nephritic and reproductive syndromes (Brown et al., 1987; Cook & Huggins, 1986; Cowen et al., 1987).

IBC often occurs in association with other viral and bacterial infections.

Their timely diagnosis is essential for maintaining the health of people and bird populations as well as for boosting the country's economy.

The main economic losses occur due mainly to three factors including the costs of drug treatment, a slowdown in the growth rate of birds and high mortality.

Nowadays, there is also another risk because of the possibility of migration of disease to the human population due to hypermutability of the pathogen.

Diagnosis of IBC is carried out taking into account the clinical signs of the disease, pathological changes that are inherent in this pathology and laboratory studies necessary for the complete identification of the causative agents of the disease.

The diagnosis of associated infections in poultry farms becomes even more complicated and requires further research.

Materials and methods

The material for our research was 125 sick chickens of ROSS 308 breed of 3-20 days of age, which were bred from chicken embryos obtained from specific pathogen free (SPF) farms.

The birds were placed in cages with feeders and drinkers and kept according to zoo sanitary standards.

Sick and dead birds were examined by clinical, pathological and histological methods. The mucous membrane of the nasal cavity, nasal septum, larynx, trachea, lungs, and reproductive organs were taken for histological examination.

The material was fixed in 10% neutral formalin, and cryosection has been done. Thin sections of kidney and trachea tissue with a thickness of 2-3 μm were cut, placed in cassettes, closed and embedded in paraffin after fixation. Then, the cassettes were fixed in a microtome, 5 μm thick slices were cut and placed on glass slides. They

were stained with hematoxylin-eosin, for fibrin according to Shueninov, for collagen fibres and fibrin according to Slinchenko, and for mucus with Meyer's mucicarmin.

After staining, the sections were dried at room temperature and a drop of Canadian balsam was applied to them. Then, they were covered with a cover glass, and examined under a microscope.

Pathological and histological changes in the structure of the trachea were assessed using a 4-point scale (Alvarado et al., 2002):

1 point - complete absence of pathological and histological signs;

2 points - indication of slight hyperplasia of the epithelium and mild subepithelial lymphoid infiltration, with a slight thickening of the mucous membrane as a result of edema;

3 points - the presence of moderate hyperplasia of the epithelium with loss of cilia and moderate subepithelial lymphoid infiltration with moderate thickening of the mucous membrane;

4 points - the presence of severe hyperplasia of epithelial and subepithelial tissue, the presence of lymphoid infiltration with moderate thickening of the mucous membrane, all cilia disappear from the surface of the epithelium and desquamation of epithelial and secreting cells of the mucous membrane occurs.

Research results

We registered the associated course of IBC with escherichiosis in the poultry farms of the republic.

We observed the associated infection of IBC with escherichiosis in young animals from one day to 10 months of age, is often less in older birds.

The contamination of chickens with IBC and escherichiosis was recorded in farms in case of violation of the manning of production groups of birds, as well as in violation of the technology of keeping and feeding.

At the same time, a reduction in the incubation period from several hours to 10-24 hours was observed.

The clinical course of the infectious process is acute or septic.

The acute course was accompanied by a respiratory syndrome: coughing, sneezing, tracheal wheezing and nasal discharge (Figure 1).



Figure1. Acute course of IBC

Wet eyes and swollen sinuses were common. Mortality in the 80% of cases was recorded mostly in an associated course with escherichiosis. The duration of the disease was 8-12 days in this case. Profuse diarrhea leading to the death of the bird was noted. In some cases, shortness of breath, paresis, paralysis, exhaustion (with a mixed course with escherichiosis) were noted.

The main pathological changes were noted in the respiratory organs. There was a sharp congestive hyperemia of the mucous membrane in the nasal cavity. Focal necrotic overlays, often localized on the vestibule and the lateral or ventral nasal passages, less often on the mucous membrane of the nasal septum, were a grayish or grayish-yellow color. There are accumulations of grayish foamy fluid on the surface of the mucous membrane in the larynx and trachea. The membrane is hyperemic. Pneumatocysts darken or may contain yellow curd exudate. (Figure 2)

Caseous plugs can be found in the lower part of the trachea or bronchi of dead chickens. Pneumonia was recorded. The lobes of the lung were increased in volume and firm consistency. Serous-fibrinous edema and fibrinous deposits were observed. The coligranulomatosis, characterized by the development of specific granulomas on the internal organs and skin, was additionally noted in some chickens. Young

animals are affected from the moment of birth, sometimes later up to 2-5 months of age or maturity. Knotty formations were found in the liver and in the intestines (with a joint course with escherichiosis) during palpation of birds.

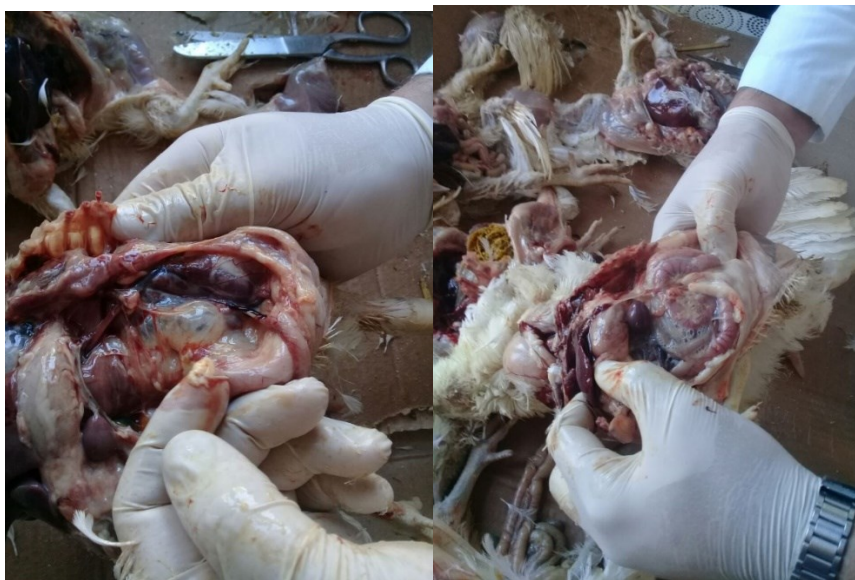


Figure 2. Pneumatocysts containing yellow curd exudate

Reproductive syndrome and a sharp decline in productivity were observed in the adult parent-flock. A sharp decrease in productivity was observed after reaching 35-50% of egg production (Figure 3).

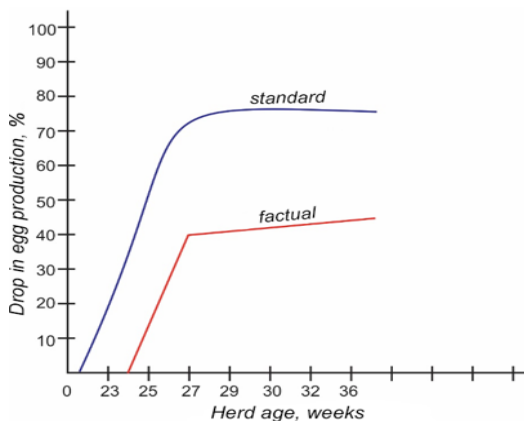


Figure 3. Curve of egg production in a population of chickens infected with IB virus and *E. coli*. No peak in productivity after reaching 50%.

The appearance and quality of eggs change: eggs are small, pale in color, almost white, sometimes the shell is soft, deformed or with lime deposits on the surface (Figure 4).

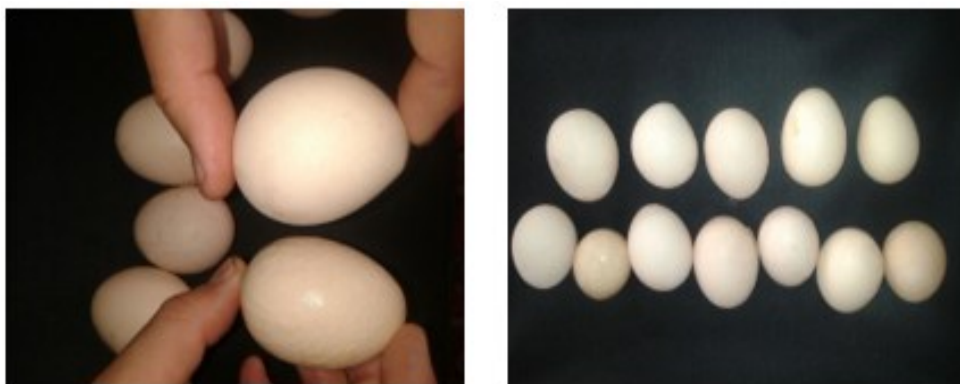


Figure 4. Curve of egg production in a population of chickens infected with IB virus and *E. coli*. No peak in productivity after reaching 50%.

An increase in the number of eggs not suitable for incubation and a decrease in the number of hatched chickens should be taken into consideration. Also, less damage to the oviduct in adult chickens was observed.

The nephrotic and nephritic syndrome was characterized by structural changes in the epithelial cells of the renal tubules, which was accompanied by a deterioration in water-salt transport and led to acute renal failure (anemia and nephritic-nephrosis syndrome were registered). We observed regeneration of the epithelium of the collecting ducts and ureters in nephritis on the 6-12th day after infection. Focal sites of uric acid deposition were recorded in swollen and pale kidneys and ureters.

Tissue regeneration was observed on the 15th day after an illness in adults.

Histological examination revealed changes 4-5 days after infection.

IB virus of R5 strain caused degeneration, vacuolization and desquamation of epithelial cells of the renal tubules. The lesion of the tubules was more established in the medulla of the kidneys. Foci of necrosis were observed in some parts. As the condition of the birds improved, the tubule epithelium is regenerated. Degenerative changes were observed when isolating the Masas strain. As a result of these degenerative changes, severe atrophy of one or two or three kidney sites happens at once.

Experimental infection of chickens causes a slowdown in the growth and loss of cilia of epithelial cells, dilatation of tubular glands, infiltration of lymphocytes, mononuclear cells, plasma cells, as well as edema and fibroplasia of the proper layer of all parts of the oviduct.

So, the clinical signs of IBC and its mixed course with escherichiosis, pathological changes and histological examination allow us to establish a preliminary diagnosis.

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History and Future Prospects of Gazelle (*Gazella Subgutturosa* Guld, 1780) Population on the Island of Khara Zira

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Abstract

The article considers the history of development of gazelle population released on the island of Khara Zira in 1965. Natural forage resources of these antelopes were estimated, factors influencing the dynamics of the gazelles population are studied, future prospects of this population are discussed and finally suggestions are made.

Keyword: *Gazella*, Khara Zira, reintroduction, European rabbit, biomass

Introduction

The distribution and number of gazelles in the south-east Caucasus depends on a number of natural and anthropogenic factors such as: fluctuation of the Caspian Sea level, expansion and shrinkage of Kura-Araz riverbeds, changes in vegetation, economic and hunting activities of nomadic and local tribes, etc. (Vereshagen, 1959).

Number of gazelles, counted in thousands at the end of 19th century, declined tenfold towards the beginning of 20th century. The number of gazelles decreased tragically after the advent of motorized transport and the cultivation of gazelles' habitats in the Kur-Araz lowlands for agricultural purposes and the occupation of habitats by human settlements. According to records of the 1960s, less than 200 gazelles remained in our country (Safarov, 1961).

One of the first steps taken by the government to save this species was the establishment of the Bandovan sanctuary and the establishment of a gazelle breeding center on the Khara Zira island (formerly Bulla). Due to the reserves, sanctuaries and

national parks were created later, and the number of gazelle herds increased to 7-8 thousand (Sarukhanova, 2016, 2017, 2019). In addition, the government started the process of returning the gazelle to its historical habitats, and the process of reintroduction is successfully continuing in several parts of the country (Sarukhanova, 2017). In this regard, the gazelle population on the island of Khara Zira retains a potential natural nursery role in the process of restoring the historical range of this species.

The purpose of the study was to monitor the population dynamics of gazelles on the island of Khara Zira, their forage base and accordingly, how many gazelles can live on the island in natural conditions, and the prospects for future use of this population in the restoration of the historical habitat of gazelles.

Study area



Figure 1. Satellite image of the Khara Zira island

Khara Zira Island is the largest of the Baku archipelago and covers an area of 400 hectares (N 39°59'43'' and E 49°38'38''). The island is of volcanic origin, consisting of a volcanic crater, a crater dam and a 1.5 km extension southwestward (Figure 1). The crater area is 30 m above the sea level. The edges of the mountain consists of

small hills, and the middle part is flat. The plain is bare as a result of volcanic activity, and it is surrounded by sparse bushes. The main vegetation of the island is semi-desert type, and the areas are not covered by volcanic mud along the coast, there is a unique desert vegetation. The island is home for wormwood, wormwood-halophytes, halophytes, ephemeral communities and their different combinations.

Materials and methods

In order to study the forage base of gazelles, the island was visited 7 times in different seasons between 2017-2020, and observations were made on routes throughout the whole island. Some plant species were identified on the site, herbariums were prepared to identify suspected species, and species affiliation was determined later.

During the counting of gazelles, the research team was divided into two groups and moved from the opposite borders of the island to the center, recording all animals encountered and taking their photos for further identification. Although it was not possible to determine the exact number of fleeing gazelles with binoculars (Nikon 10x42), further clarifications were made on the photos taken applying the computer techniques.

Results and Discussion

During our research, a clear seasonality was observed in the development of vegetation on the Khara Zira island, i.e. intensive spring vegetation disappears in early May and stops completely in summer. After the rains in the fall, there is a revival and vegetation is restored, and this lasts all winter due to the mild and snowless winter (average temperature is 8-10⁰C). The vegetation of the coastal strip and the island is sparse and consists of halophyte groups, especially the Herbaceous sea-blite (*Suaeda maritima ssp. Rostrata*). In some humid sands *Juncus littoralis* grows.

Wormwood semi-desert phytocenosis is observed at intervals along the coast. These phytocenoses represented by *Artemisia lercheana* (synonymous is *Artemisia fragrans*), with halophyte - *Salsola nodulosa*, and ephemeral plants - Cereals, Brassicaceae, Boraginaceae, Asteraceae, etc.

Although ephemeral plants found in the form of islets are not tall due to the high salinity and dryness of the soil, they are the main food for gazelles during the rainy seasons. As wormwood and ephemerals burn completely during the summer months,

they are partially replaced by the camelthorn (*Alhagi pseudalhagi*) and are one of the few forage plants for gazelles. In August, the stems of this plant are completely eaten by gazelles. In times of drought, one of the gazelles' prey is the bark of the *Tamarix ramosissima* shrubs, which are found along the island. After the lifeless area around the volcanic crater, there are small halophytic shrub deserts.

Semisrub species, such as *Halocnemum strobilaceum* and *Salsola ericoides*, were widespread here. Rarely *Salsola dendroides* is also found on the island. Although halophyte formations are low-calorie foods, they are the main nutrition source for animals on the island during the winter. The list of plants identified from the island during previous studies is as follows: *Anisantha rubens*, *Artemisia szovitsiana*, *Suaeda cf dendroides*, *Alopecurus myosuroides*, *Anisantha rubens*, *Artemisia cf lerchiana*, *Artemisia szovitsiana*, *Ephedra distachya*, *Filago pyramidata*, *Herniaria hirsuta*, *Hordeum spec.*, *Koelipinia linearis*, *Lolium rigidum*, *Parapholis incurva*, *Poa bulbosa*, *Sonchus oleracius*, *Suaeda cf dendroides*, *Tamarix hohenackeri*, *Kalidium caspicum*, *Psylliostachys spicata*, *Lycium ruthenicum*, *Salsola dendroides*, *Ephedra distachya*, *Kalidium caspicum*, *Psylliostachys spicata*, *Suaeda cf dendroides*, *Silybum marianum*, *Tamarix ramosissima*, *Tamarix tetrandia*, *Artemisia cf lerchiana*, *Kalidium caspicum*, *Anisantha rubens* (Annett Thiele *et al.*, 2008).

During Soviet period, some attempts were made to calculate the carrying capacity of the island. If we take the biomass of the island's vegetation into consideration, it is possible that 70-80 head of gazelles live here due to natural fodder reserves, and in case of additional feeding, this figure can reach 150-200. Thus, considering the absence of predators on the island and exploiting the area as a natural nursery, it is possible to capture 30-50 gazelles per year and relocate them to other areas within the historical range of gazelle (Jafarov, 2008).

35 gazelles (20 females and 15 males) were first brought to Khara Zira Island in 1965. Due to the island's lack of natural fodder reserves, the number of gazelles has increased up to 120 in 11 years as the result of additional feeding (96 dead individuals are not brought into record). As the supplementary feeding was not economically sustainable, gazelles were caught on the island and taken back. 30 gazelles captured on October 7, 8 and 24 in 1976 were released into the Absheron sanctuary and 70 gazelles into the Shirvan reserve. Thirteen gazelles remained on the island at that time, the number of them reached 18 in 1983 and decreased to 9 gazelles a year later. Poaching has been cited as the reason for the decline (Jafarov, 2008).

Further research on the island of Khara Zira was conducted by the Michael Zukkov Foundation in 2007-2008, and 15 gazelles were still living there (Annett Thiele *et al.*, 2008).

For the first time on August 12-13, 2017, during the trip organized by the Institute of Zoology of ANAS, we studied the number of gazelles that survived on the island, their food reserves, threats to them, and we counted 15-18 gazelles there. At the same time, we have observed a massive increase in the number of rabbits (*Oryctolagus cuniculus*). The introduction of rabbits to the islands in the Caspian Sea was carried out by sailors and lighthouse keepers in the 19th century as a reserve food (Hajiyev, 2000). Although they became extinct on many islands by the end of the 20th century, they were re-released by the inhabitants of the surrounding settlements and fishermen. According to our estimates, there were more than a thousand individuals on the island in 2017, and they were in serious food competition with gazelles.

During the field research (09, Sep 2018), we observed the mass mortality of rabbits, and 30 dead rabbits were recorded along the 8 km transect. The cause of death could be both malnutrition and disease. According to fishermen, one of the main threats to gazelles is still poaching.

Our surveys on September 13, 2019 showed that the number of gazelles is already around 25-30. During our study, a significant decrease (about 10 times) in the number of rabbits were observed by us. We assume that the frequent visits to the island in the last two years and the enlightening conversations with the fishermen who have temporarily settled here have also had a positive impact and poaching was decreased.

During our last survey conducted on July 2, 2020, it was estimated that only 11 gazelles remained on the island. Another disappointment was that a single offspring was observed that year. The reason for the fact that the number of gazelles has more than halved in the last year can be explained by the drought observed in recent years (Fig. 2). Vegetation on the island has actually been reduced and destroyed by drought. It has been observed that there is still no increase in the number of rabbits and that they are rare along the transects. During the quarantine period declared in the country due to the COVID-19 pandemic, outsiders (fishermen, poachers, etc.) were not allowed on the island, and the role of the human factor is unlikely in such a sharp decline in the number of gazelles.

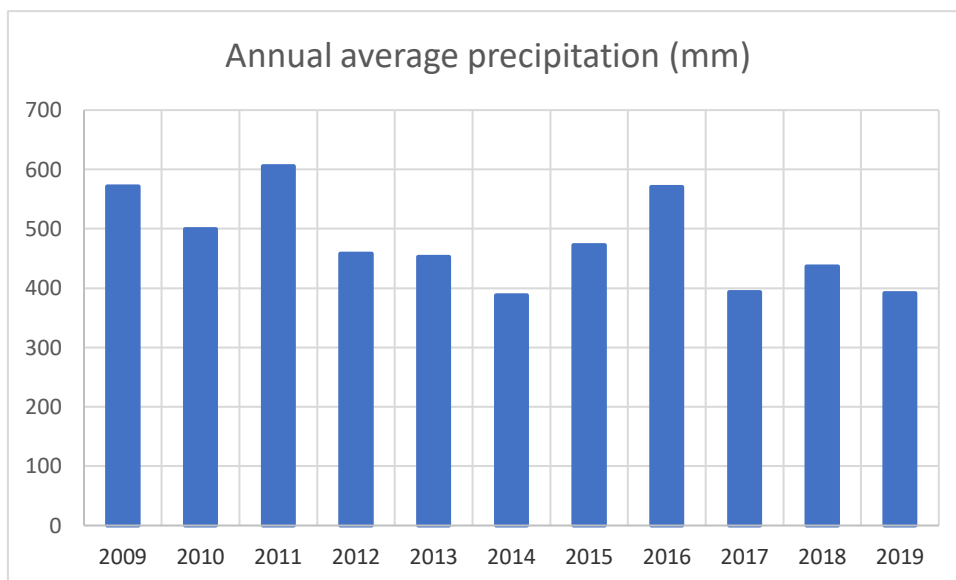


Figure 2. Annual average precipitation in Azerbaijan
(www.stat.gov.az/source/environment/)

Conclusions

We agree with conclusions of Soviet scientists stating that the natural resources of Khara Zira Island can allow up to 70-80 gazelles to live here. With additional feeding conditions, the number of gazelles can be increased up to 150, where they do not have any natural enemies. Later, at least 25-30 individuals can be reintroduced to other parts of the country within the programs on restoration of gazelle's historical range. The reason for the decline in gazelle food stocks is the massive increase in the number of rabbits introduced here and also an arid climate.

Therefore, the following further actions are strongly recommended:

- to establish a real protection regime on the Khara Zira island,
- to plant the seeds of the species on the island which can serve the main food and water source for gazelles (cereals, wild watermelons etc.)
- to introduce common weasel (*Mustella nivalis*) individuals in order to control the rabbit population. Bringing larger predators to the island can be dangerous for newborn gazelles.

- in order to fertilize the soils on the island, it is necessary to introduce coprophage insects (dung beetles - *Geotrupes* spp). Our observations show that since the excrements of rabbits does not mix with the soil, they are crumbled by the sun and rain, and then blown into the sea by the winds, and the soil of the island is not fertilized. Dung beetles cause soil fertilization because they bury manure masses.

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Dynamics of Associative Infection of Domestic Chickens with Eimeriosis and Infectious Diseases in Azerbaijan

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Abstract

The article refers to the associative infection of birds with invasive and infectious diseases in the poultry farm of the Siyazan region. In order to determine the infection with *Eimeria*'s oocytes and opportunistic microbes of domestic chickens, pathological materials from 35- and 55-day-old chicks of domestic chickens of a silver breed were used. As a result of the study, the following causative agents were identified in the carcasses of 35-day-old birds: *Escherichia coli* in 11 birds (17.0%), *Salmonella enteritidis* in 28 birds (43.0%), *Staphylococcus pyogenes aureus* in 4 birds (6.1 %).), *Streptococcus faecalis* in 3 birds (4.6%), and out of invasive diseases the following causative agents of eimeriosis were identified: *Eimeria acervulina* in 5 birds (7.7%), *Eimeria tenella* in 8 birds (12.3%), *E. mitis* in 6 birds. (9.2%), *E. maxima* in 5 birds (7.7%).

When examining the carcasses of 55-day-old birds the causative agents of infectious diseases: *Escherichia coli* in 10 birds (16.6%), *Salmonella enteritidis* in 20 birds (33.3%), *Staphylococcus pyogenes aureus* in 3 birds (5.0%), *Streptococcus faecalis* in 6 birds (10.0%), and out of invasive diseases the following causative agents of eimeriosis: *Eimeria acervulina* in 4 birds (6.6%), *E. tenella* in 7 birds (11.6%), *E. mitis* in 5 birds (8.3%) and *E. maxima* in 3 birds (5.0%) were studied.

Keywords: domestic chickens, invasion, infection, mixed infection, examination

Introduction

Poultry farming is one of the important sectors in providing the population with food products. It is known that for a long time poultry farming existed in households, and only since recently, it has formed and developed as an independent branch of agriculture.

Special attention and care is paid to the development of this area in our country. Therefore, today poultry farms have got a special/vital/critical role in providing the population of the region with poultry meat and eggs through domestic production. Along with the measures taken to increase the production of poultry meat and eggs in households and small businesses, modern poultry farms are being created and old ones are being reconstructed, owing to preferential loans provided by the state.

In connection with the intensification of poultry farming as a fast-growing and economically profitable sector in the second half of the last century, poultry factories, focusing on the production of meat and eggs, were created in Azerbaijan. Having gained the Republic independence, these farms were privatized and developed in compliance with economic reforms.

As a result of the transition of poultry farming to a farm basis, the epizootic situation for infectious and invasive diseases, especially for the ones caused by opportunistic microbes and eimeria, has changed significantly (Mikailov & Mamedova, 2014). Thus on the one hand, in the poultry farms, , due to the accumulation of birds of different ages in large numbers in a limited area and their regular renewal, deficiencies in the conditions of feeding and keeping, the even greater sensitivity of fertile crosses to stress factors and diseases, on the other hand, due to the cessation of the development of the beneficial microflora of the gastrointestinal tract in connection with the administration of drugs to chickens, the general condition and natural protective functions of the bird's body weaken from the first day. (Musayev et al., 1991). In this case, the immune balance between the microorganism and the macroorganism is disturbed, leading to creating favorable conditions for the development of pathogens of infectious and invasive diseases, and as the result the occurrence and spread of the disease. In such condition, the disease is diagnosed using diagnostic methods (Nakhmanson & Burba, 1990).

The mixed course of diseases such as salmonellosis, colibacillosis, staphylococcosis, streptococcosis and out of invasive diseases, the eimeriosis caused by opportunistic microbes causing economic damage to poultry farms, an epizootic situation, identification of factors, creating conditions for their occurrence, as well as their spread, control measures against them are being studied (Borisenkova et al., 2011; Mikayilov, 2014; Mirzakov & Tashbulatov, 2011; Lesnichenko & Emaluev, 2011). Hence, through our research, we set a goal for ourselves, to study the occurrence in an associative form of infectious and invasive diseases (eimeriosis).

Materials and methods

The studies were carried out in 2019 on the basis of pathological materials brought from the poultry farm from the Siyazan region. Microbiological studies were carried out in the department of avian diseases of the AzVSRI, and scatological studies were performed in the department of parasitology. The carcasses of the birds were examined by means of pathologico-anatomic autopsy, and diseases caused by opportunistic microbes and the causes of death on the farm were revealed. The experiment has revealed the changes characteristic of colibacillosis, salmonellosis, staphylococcosis and streptococcosis. In order to confirm the diagnosis, meat peptone broth (MPB) and meat peptone agar (MPA) from the tubular bone marrow of bird carcasses were inoculated into a nutrient medium and after incubation for 24 hours at 37° C, the condition of microbe's growth in a nutrient medium was checked, and transferred from MPB where the changes took place into the Endo nutrient medium, which is the medium for differential diagnosis. The main purpose of using the bismuth sulfite culture medium was to determine if the microbial culture isolated after 20-24 hours in Endo food medium was from the E. coli group or the Salmonella group. Saline and bloody MPA from the selected nutrient media were used for isolation and study the cultures of Staphylococci and Streptococci.

Materials (samples of faeces) collected for determining the presence of Eimeria in birds were examined scatologically using the Darling-Fullborn method. The samples were centrifuged at 1500 rpm for 5 min. A solution of saturated sodium chloride was added to the sediment in a ratio of 1:10, got mixed, and then centrifuged again. A small drop was taken from the upper layer of the suspension using a metal loop, examined and observed through a microscope, and infection was determined. Postmortem examination of dead bird carcasses revealed hemorrhages in the intestines, and microscopic examination of scrapings taken from the intestines revealed Eimeria oocysts.

Results and discussion

Keeping and increasing the productivity of birds depends on the well-being of poultry farms in regard with infectious and invasive diseases, on disease and death of birds, the timely identification of the reasons for the drop in productivity, and on the development and implementation of scientifically grounded measures to control them.

It should be noted that after the transition of poultry farming to a farm basis, and as per our research, we have determined the epizootic situation of the disease, the

spread of the disease, the factors contributing to their occurrence, the peculiarities of the mixed course of infectious and invasive diseases. To determine the reasons of the diseases caused by opportunistic microbes and eimeria, and the causes of mortality of birds at the poultry farm, postmortem examinations were carried out in more than 250 bird carcasses. As a result, characteristic changes of colibacillosis, salmonellosis, streptococcosis, staphylococcosis and eimeriosis were revealed. To confirm the diagnosis, bacteriological and scatological examinations of tubular bone marrow, feces samples, and intestinal scrapings were carried out in 65 carcasses of 35-day-old birds and 60 carcasses of 55-day-old birds. As a result of the study, we isolated the causative agents of the above diseases from the carcasses of 35-day-old birds. Out of these study groups/cases, we detected the results as follows:

Escherichia coli in 11 birds (17.0%), *Salmonella enteritidis* in 28 birds (43.0%), *Staphylococcus pyogenes aureus* in 4 birds (6.1%), *Streptococcus faecalis* in 3 birds (4.6%), *Eimeria acervulina* in 5 birds (7.7%), *Eimeria tenella* in 8 birds (12.3%), *E. mitis* in 6 birds (9.2%), *E. maxima* in 5 birds (7.7%) were detected. *Escherichia coli* in 10 birds (16.6%), *Salmonella enteritidis* in 20 birds (33.3%), *Staphylococcus pyogenes aureus* in 3 birds (5.0%), *Streptococcus faecalis* in 6 birds (10.0%) from the tubular bone marrow of 55-day-old bird carcasses and *Eimeria acervulina* in 4 birds (6.6%), *E. tenella* in 7 birds (11.6%), *E. mitis* in 5 birds (8.3%) and *E. maxima* 3 in birds (5.0%) from intestinal pruritus, and during the scatological examination of samples of faeces *Eimeria acervulina* in 4 birds (6.6%), *E. tenella* in 7 birds (11.6%), *E. mitis* in 5 birds (8.3%) and *E. maxima* 3 in birds (5.0%) (Table 1).

Thus, bacteriological and scatological examinations of pathological materials of 135 dead chicks revealed opportunistic pathogenic microbes and eimeria. Our research plays a key role in determining the prevalence of infectious and invasive diseases (colibacillosis, salmonellosis, staphylococcus, streptococcosis and eimeriosis) in poultry farms. As a result, research will have a positive impact on the profitability of farms by preventing the level of microbial contamination of hatching eggs, incubators, feed and air in poultry buildings. Therefore, it is important for farms to comply with the requirements of veterinary and sanitary regulations at all stages of production.

Table 1. Results of examination of pathological materials

Age of birds	Examined	Causative agents		35-day old	55-day old
		Infected	%		
35-day old	Examined	Infected	<i>Escherixiya coli</i>	11	17,0
			%	28	43,0
	Examined	Infected	<i>Salmonella enteritidis</i>	4	6,1
			%	3	4,6
	Examined	Infected	<i>Staphylo-coccus pyogenes aureus</i>	5	7,7
			%	3	4,6
	Examined	Infected	<i>Strepto-coccus faecalis</i>	5	7,7
			%	8	12,3
	Examined	Infected	<i>Emmeria aceru-lina</i>	6	9,2
			%	7	11,6
	Examined	Infected	<i>Emmeria tenella</i>	6	9,2
			%	5	7,7
	Examined	Infected	<i>Emmeria mitis</i>	5	7,7
			%	3	4,6
	Examined	Infected	<i>Emmeria maxima</i>	3	4,6
			%	5	7,7

Conclusion

1. During the examination of 35-day-old bird carcasses, the following causative agents of infectious diseases were detected: *Escherichia coli* in 11 birds (17.0%), *Salmonella enteritidis* in 28 birds (43.0%), *Staphylococcus pyogenes aureus* in 4 birds (6.1%), *Streptococcus faecalis* in 3 birds. (4.6%), *Eimeria acervulina* from 5 birds (7.7%), and out of invasive diseases, the causative agents of Eimeria: *E. tenella* in 8 birds (12.3%), *E. mitis* in 6 birds (9.2%), *E. maxima* in 5 birds (7.7%).

2. Examination of 55-day-old bird carcasses has identified the following causative agents of infectious diseases: *Escherichia coli* in 10 birds (16.6%), *Salmonella enteritidis* in 20 birds (33.3%), *Staphylococcus pyogenes aureus* in 3 birds (5.0%), *Streptococcus faecalis* in 6 birds. (10.0%), and out of invasive diseases, the causative agents of Eimeria: *Eimeria acervulina* in 4 birds (6.6%), *E. tenella* in 7 birds (11.6%), *E. mitis* in 5 birds (8.3%) and *E. maxima* in 3 birds (5.0%).

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Application of the Hydroponic Green Fodder Technology in Poultry Breeding and Maintenance of the Broiler in as Provided by Zoogygienic Conditions

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Abstract

The article sets out as a goal the ensuring in a sustainable form of innovative development in the designing of feeding strategies within the framework of the project "Sustainable development of poultry farming and the creation of a value chain for food production" in farms of the republic. Also, for rational and proper feeding of birds, the use of the Hydroponic Green Fodder technology, the presence of high-calorie protein (raw protein) in the feed, as well as amino acids, a small amount of cellulose, carotene, calcium and phosphorus, create favorable conditions for the development of chickens.

Keywords: hydroponics, rational feeding, technology, raw protein, infection

Introduction

The development of breeding and feeding strategies under the “Sustainable Development of Poultry Farming and Creation of Value-Added Chain for Production of Foodstuffs” Project, funded by the Government of Azerbaijan and have been implementing since December 2018 under the FAO-Azerbaijan Partnership Program to ensure production in line with market demand, was set as a goal to develop in a sustainable manner the functioning of added value chain for poultry.

Since poultry farming is one of the fast-growing, widespread and profitable sectors of animal husbandry, the government's concern for the efficient use of this industry has increased significantly. Thus, in the field of poultry farming, new farms and individual farms have been created. Relevant work has been carried out in the direction of increasing poultry production using hydroponic and other innovative technologies. The increase in financial support for the development of the poultry farming from year to year, the commissioning of production sectors involved in the

production and packaging of poultry meat and the marketing organization have played an irreplaceable role in the development of this sector (Mitrofanov, 2010).

The development of poultry farming, as well as an increase in the output of poultry farming products, largely depends on the fact that the chickens selected for raising of broilers are healthy, lively, well developed and of the same weight. That is, if there is a weight of 55-60 grams of eggs laid in the incubator, the weight of one-day-old chicks is 35-40 grams. Healthy chickens hatch on time and in droves, grow quickly, and mortality decreases. Healthy chicks are selected in 6-8 hours after hatching, and the retarded chicks from growth are culled (Zayas, 2013).

For the proper feeding of birds, the Hydroponic Green Fodder technology is one of the key factors in ensuring the intensive growth of chicks. Because of the presence in the feed allowance consisting of high-calorific protein (raw protein), as well as amino acids, a small amount of cellulose, carotene, calcium and phosphorus, creates favorable conditions for the development of chickens (Mikhailov, 2014).

Innovation of Hydroponic Green Fodder technology

The introduction of innovations in farmers' poultry units plays an important role in the modernization and intensification of production. Therefore, there is a need to apply an innovation mechanism for improvement the feed supply in farmers' poultry units. From this perspective, the innovation of Hydroponic Green Food technology for improvement the feed supply in poultry farms can meet the needs of not only small farm enterprises, engaged in poultry farming, but also of large complexes. From this point of view, there are no analogues to the Hydroponic Green Fodder technology. Using this method, in agriculture, regardless of weather conditions, it is possible to produce green fodder of steady quality, continuing during 365 days a year. Grains sprout at an above-zero temperature of 18° C, and micro-macro vitamins are transferred to the plant along with water. So long as, green fodder is rich in high-calorific protein (raw protein), as well as amino acids, carotene, calcium, phosphorus and vitamin E, which leads to increased productivity indicator in poultry. The feed obtained by this method improves digestion in birds and stimulates weight gain (Mikhailov et al., 2017).

Birds eat up this food with great appetite. It should be considered that 1 kg of green fodder contains 20-25% of raw protein, 4-5% of fat, 35-50% of nitrogen-free extraactive substances, 9-11% of mineral salts and 10-15% of cellulose. Plus, 1 kg of green fodder contains 40-50 mg of carotene, which directly affects the productivity of birds (Mikhailov et al., 2017).

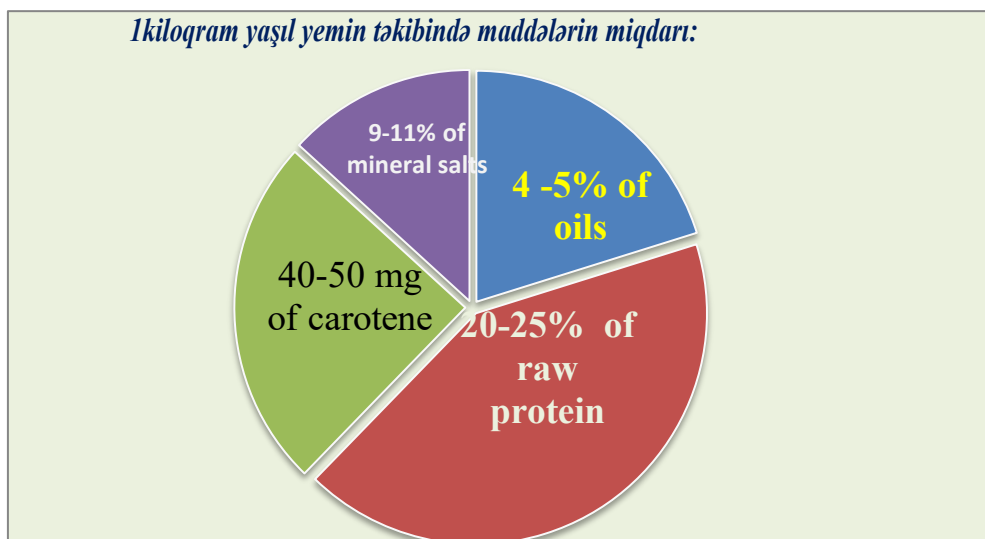


Figure 1. The amount of substances in the composition of 1 kg of green fodder

As a result of the application of the Hydroponic Green Fodder technology in family poultry farms, in 2019, the total number of eggs obtained from each chicken increased by 11.6%, the weight gain (200 g) in each chicken (in broiler) intended for slaughter - increased by 13.3%, the number of chicks leaving the incubator increased by 12.2%. As a result of the use of Hydroponic Green Fodder in the feed allowance, the efficiency of the farm has increased and the costs of feed for each bird has decreased. The complete organization of the sprouting of green bird feed by the hydroponic method takes 5-6 days. Thus, from 1 kg of wheat within 5-6 days, 4-5 kg of green mass is obtained. It should be noted that wheat is first washed in special containers, this clears the grains from poisonous spores of fungi and dust, and then the grains for sprouting are laid out in each container at a rate of 1–1.2 kg per one container. Starting from the second day, seedlings are obtained. On the third day, the root system of the grain begins to branch out and develop vegetatively. On the fourth to fifth days, along with the rapid development of both roots and green mass, the amount of raw protein (protein), starch (sugar), mineral salts, etc. is increased in the composition of the feed. Therefore, from an environmental point of view, with regard to the full practical value of pure fodder, it is used to meet the needs of poultry for raw protein, water carbon, calcium, phosphorus and carotene. Birds eat food along with the roots, since the main nutritional value is in the root system. Continuous feeding with hydroponic green fodder ensures the health of broilers, daily weight gain, and the development of quality poultry meat production.

In order to ensure the innovative development of broilers, the proper formulation of the feed allowance of chickens creates the opportunity for rapid growth of the broiler

and hence the presence of a high percentage of healthy rearing. Demand for feed depends on the age and body weight of the broiler.

For the first five days, chicks are fed on boiled eggs, wheat bran, bottom milk, fresh anthroponic green fodder, crushed shells, and so on. Eggs should be boiled in clean water, then crushed together with the shells and given as feed. It is also recommended to add additionally 2-3% of feed yeast to feed. In this case, the yeast increases the amount of vitamins in the feed by increasing the number of cells and bacteria. The normal metabolism in young birds is largely depended on the good care of chicks. Thus, the soaked feed mixture is fed to 30-day-old chickens 3-4 times a day (one ration of hydroponic green fodder), and then 2-3 times. Chicks up to ten days of age are fed every two hours, and then up to 30 days of age every 3 hours. For each head of broiler in the first week, 12-15 g are given, 21-20 g in the second week, 40-45 g in the third week, 60-65 g in the fourth week, 75-85 g in the fifth week, 90-100 grams in the sixth to seventh weeks and after the eighth week 100-110 grams of feed are given. The live weight of a broiler fed in this way for seven to eight weeks with the consumption of 2-2.5 kg of a feed unit per 1 kg of live weight is 1500-1600 g and more.

By a long-term feeding with compound feed, for each ton of it, 10-15 grams of antibiotics should be added. The antibiotics given at this dose have a stimulating effect on the rapid growth of broilers and prevent many intestinal diseases. On the other hand, long-term use of antibiotics with high doses retards the growth of broilers, deteriorates the composition of some vitamins and gives poor results. All antibiotics should be removed from the feed ration 8-10 days before slaughter. After 49-63 days, broilers are transferred to the slaughtering and processing shop. For 6-8 hours prior to the slaughter, broilers are kept without feeding (Shafiyeva, 2005).

Breeding of broilers on bedding

During the rearing on the bedding, 4-5 batches of chickens intended for meat can be raised in one premise per year. After breeding of each batch, the premise is cleaned, disinfected, the bedding is laid a new and the next batch is accepted. The following technology is used/applied on a thick bedding: 0.7-1 kg of slaked lime is laid on each plot of a square meter, corn stalks in 5-7 cm of thickness, peat, tree bark or chopped straw are laid on it. Until the end of the breeding period, 1.5-2.0 kg of bedding is used per bird. During the breeding period, 10-12 chickens are kept on each square meter in winter, and 9-10 chickens in summer (Shafiyeva, 2005).

Effect of aeration on breeding of broilers

In broiler development, air exchange is of great importance. With insufficient oxygen in the premise, the chickens intended for meat eat less than usual, respiratory diseases develop among them, and the expected weight gain is not achieved. Therefore, ventilation must be regulated in such a way that for each kg. of live weight there was produced 1.5 cubic meters of air exchange. In addition, when the premise where the broilers are bred is lit for 18 hours in the first week during the day and then gradually reduced to 14 hours. Good results can be obtained in increasing the weight of meat chickens. Since chicks are unable to regulate their body temperatures from day one, temperature fluctuations inside the premise will have a sharp effect on them. The presence of a sharp air flow inside the premise causes such abnormalities in birds as diarrhea, delayed feathering, and deterioration in digestion. Cold and wet weather causes kidney damage, wet and cold bedding causes severe diarrhea, and dust, ammonia and carbon dioxide leads to the occurrence of various diseases of the respiratory organs. At normal heat levels, chicks are in brisk condition, their feathers are smooth, they readily eat feed, drink enough water and this ensures the rapid weight gain (Mikailov et al., 2017).

Control over the quality of water and feed used in poultry farms is of great importance. Water pollution with organic substances also leads to dangerous consequences. Poor water quality leads to corrosion, tanning and becoming moldy of some means in poultry farming as well. Water hardness depends on the amount of calcium containing in it. The presence of more than 4 mg of calcium in one liter of water indicates its hardness. In order to provide birds with good quality of water, oxidizing agents that are not harmful to birds should be added to the composition of water composition. For this purpose/to do this, citric acid and chlorine are most commonly used. In this case, the total number of microorganisms in the water decreases, the pollution with organic substances comes down, and the accumulation of some metals reduces. Drinking containers should always contain clean water. In order to protect chickens from gastrointestinal diseases, potassium permanganate is added to the water in a ratio of 1: 10,000.

Results and discussion

1. Development of breeding and feeding strategies within the framework of the "Sustainable development of poultry farming and creation of value added chain for production of foodstuffs".

2. Carrying out appropriate work/Observing appropriate principles in the direction of increasing the production of poultry meat in farm enterprises and individual farms using hydroponics and other innovative technologies.
3. To organize intensive growth of poultry, it is proposed using the hydroponic green fodder technology for full-quality and rational feeding of broiler and keeping in accordance with zoohygienic conditions.
4. When rearing a broiler, air exchange and breeding on bedding the regulation in accordance with zoohygienic standards should be controlled.
5. Entry routes to poultry-houses, hatchery houses, slaughtering shop and other premises should be equipped with disinfectant barriers/facilities 1.5 m long and 15 cm deep, which should be regularly moistened with disinfectant solutions.
6. For disinfection of automobile tires, new disinfection barriers should be organized and a 3% formaldehyde solution or chlorinated lime solution with 1% active chlorine in its composition, 5% creolin or xylonaphtha solution should be added there.

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