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# The determination of domesticated animal species from a Neolithic sample using the ELISA test

## *Détermination d'espèces d'animaux domestiques à partir d'un échantillon néolithique grâce au test Elisa*

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ABSTRACT

This is a report on the successful application of Enzyme-Linked ImmunoSorbent Assay (ELISA) in archaeozoology, particularly for the taxonomic determination of severely fragmented bone material from archaeological contexts of the locality (Czech Republic) dated to the Central European Neolithic period (approx. 5500–4500 BC). Physical, chemical and biological features of soil deposits in the sites examined are the likely cause of the crumbly consistency of the bone material supplied to the laboratory. These factors dwarfed the ratio of specimens determinable by their physical morphology to 28%, thus limiting the reliability of inferences on the character of the economy practiced in the excavated sites. The mass spectrometric approach is the suitable standard for ancient protein investigation, but the high financial requirements prevent practical adoption of the method for fast and routine identification of bone fragments. One way to more easily and cheaply differentiate the taxons of domestic animals on the basis of bone chips is the ELISA test. In the past, experiments trying to improve the unfavourable ratio by a protein radioimmunoassay (pRIA) method yielded positive results. However, similar outcomes can be achieved by commercially available and therefore less laborious ELISA kits that were originally designed for use in inspections of the food industry.



*Mots clés :*
Elisa
Protéine
Néolithique
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Animal domestique

RÉSUMÉ

Cet article met en avant l'intérêt de l'outil Elisa (acronyme de Enzyme Linked ImmunoSorbent Assay) pour l'archéozoologie, en particulier pour la détermination taxinomique des restes de faune fortement dégradés. Il est appliqué au site archéologique de Molitorov (République tchèque), occupé au cours de la période néolithique (approximativement, 5500–4500 avant notre ère). Les conditions physiques, chimiques et biologiques défavorables du milieu d'enfouissement sont à l'origine de l'aspect friable des restes osseux mis au jour sur ce site. Dans le cadre d'une analyse archéozoologique classique, cette forte dégradation a pour conséquence un taux de détermination très faible (28 % des restes), diminuant ainsi la fiabilité des interprétations formulées sur les pratiques d'élevage néolithiques. Le

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test immunologique Elisa, qui est destiné à détecter ou à doser une protéine dans un liquide biologique, nous a permis de déterminer à l'espèce des fragments osseux de façon plus simple et moins onéreuse qu'avec la méthode de spectrométrie de masse, utilisée de façon standard pour l'identification des protéines anciennes. Cette dernière ne se prête, en effet, pas à la mise en place d'un dispositif de détermination systématique des restes de faune. Par le passé, des tentatives pour améliorer le taux de détermination à l'aide d'une protéine radio-immunologique (pRIA technique) avaient fourni de bons résultats. Toutefois, des résultats similaires peuvent être obtenus par le biais des kits Elisa, disponibles dans le commerce et plus simples d'utilisation, qui ont été conçus à l'origine pour les contrôles dans l'industrie alimentaire.



## 1. Introduction

Fragmentary bone collections from archaeological contexts pose many difficulties for the study of the past economy. When a ratio of undeterminable specimens is too high, any reliable interpretation of a sample is severely limited. Furthermore, archaeological finds provide ample examples of cultural artefacts made of animal bone which can often be changed to such an extent that their original morphology has been obliterated. Again, assessment of a source material species is usually problematic. In an attempt to avoid these problems, at least in selected cases where a more accurate and/or representative information is desirable, we have tested the possibility of taxonomical classification of bones of domesticated animal on the basis of species-specific protein detection by means of antibodies.

This approach is certainly not a novelty. Immunological tests have been applied to archaeological material in the last two decades (Cattaneo et al., 1992, 1994, 1995; Waite et al., 1997), but the main focus of bioarchaeological research has shifted to other problems. In our opinion, complete abandonment of this avenue of research would be premature. Original exploitation of protein radioimmunoassay method (pRIA) in forensic biology and archaeology (Lowenstein et al., 2006; Reuther et al., 2006) has been largely replaced by the Enzyme-Linked ImmunoSorbent Assay method (ELISA), when instead of a radioactive signal, the antigen–antibody reaction is measured by colorimetric signal.

Protein analyses can be used even for distinction of sheep and goat bones, which are usually difficult to determine on the basis of their morphology (although the test used in this study cannot distinguish these two species). Sheep and goat bones were successfully distinguished recently using a single collagen peptide (Buckley et al., 2008).

The main advantage of an ELISA test is its relative simplicity and easy application, even in the conditions of a field laboratory. In archaeology, this is an especially important aspect.

## 2. Proteins versus aDNA

In this article, we are addressing the problem of identification of domesticated animal species from fragmented bones. Commercial ELISA test kits are available for detection of even very small amounts of denaturated proteins in food. For this reason, we attempted to use these tests for the needs of archaeology. In the ELISA method, an antigen must be immobilized to a solid surface. The antigen is then complexed with an antibody that is linked to an enzyme. Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product. The most important element of the detection is a highly specific antibody–antigen interaction.

Nowadays, standard ELISA is frequently used for bacterial pathogen detection in medical research, but for the purpose of species assignment and sexing of samples it not been used much since the 1980 s. When the PCR method became widely accessible, the rapid development of appropriate DNA tests replaced ELISA in these basic tasks, arguing that DNA tests are generally faster, cheaper and more reliable. However, such evaluation holds entirely true only in the case of recent biological material. Analysing archaeological samples hundreds or thousands of years old (ancient DNA) is a task accompanied by a whole array of problems which have been recognized and discussed in publications (Bouwman and Brown, 2005; Pavelka and Šmejda, 2007, our study; Willerslev and Cooper, 2005). The natural processes of DNA degradation in the time after the death of an individual have proved to be affecting results of aDNA analysis. Thus, the limiting factor for aDNA studies is the degree of DNA preservation. The study on 291 prehistoric cattle remains from Europe, the Near East and North Africa revealed that DNA preservation is mainly influenced by geographic and climatic conditions. Especially in hot climates, the preservation of sample material is generally low (Bollongino et al., 2008b). Therefore, the benefits of DNA analysis use for ancient samples mentioned above diminish in comparison with specific antibody testing. Some proteins are more resistant than DNA against environmental degradation. Proteins offer an especially promising possibility for such evaluation (Poinar and Stankiewicz, 1999). DNA naturally bears more information in comparison to locally preserved proteins. For more detailed analyses, especially of recent material, DNA is more appropriate. Nevertheless, more resistant proteins can give a rough identification of the species, or genus of less preserved ancient samples when morphology cannot be used.

It has already been observed that a substantial difference between the results of aDNA extracted by PCR and immunochemical detection applied on ancient organic remains may occur. The explanation for this may rest in the fact that hydrolytic chain scission events have a much more


**Fig. 1.** The bone artefact tested (bone bodkin).
**Fig. 1.** Artefact osseux (poinçon) testé.

destructive impact on the long sequences of DNA required for PCR than on the numbers of shorter antibody binding sires detected/used by immunochemistry (Waite et al., 1997).

Hence, antibodies still remain a useful detection technique in archaeological science. We can mention several examples of recent research projects, which benefited from the application of closely related methods aimed at ancient proteins. Using Western blots by specific antibodies, proteins from the immune system and collagenous ancient proteins were detected (Schmidt-Schultz and Schultz, 2004). ELISA analysis was used to detect the enteric parasites of two medieval latrines (Mitchell et al., 2008). Investigation of protein sequences of fossil hominids (Neanderthals) brought results, e.g. for osteocalcin (Nielsen-Marsh et al., 2005). Immunodetection and quantification were successful in non-collagenous proteins extracted from archaeological samples of bone material (Brandt et al., 2002). Quite recently, an identification and sequenation of a 65 million-year-old protein were announced (Asara et al., 2007). However, some conclusions of the paper are controversial (Buckley et al., 2008).

## 3. Material

Bone fragments used in this analysis originated mainly from the rescue excavation carried out in a clay pit of the brick factory at Molitorov, Kolín district, Czech Republic, in the years 2005–2007 (the fieldwork was supported by the grant IAA800020503, GA AV ČR). They are dated by associated archaeological artefacts (Fig. 1) to the Neolithic LBK culture (Linear Pottery culture). The site lays at an elevation of 285 m asl, on brown soils derived from underlying loess (Šumberová and Tvrdík, 1999).

The archaeologically examined area of Molitorov is found at the edge of a Českobrodsko and Černokostelecko Permian structure on an elevated ridge between nameless water streams. According to the Geological map of the Czech Republic, part Kolín (3954), the geological bedrock, is of crystalinic schist. According to the Český Brod Geological Conditions Survey, the soil surface is of brown soil on loess and loessical soils, which lay on Tertiary weathered clay (Šumberová and Tvrdík, 1999). The brown soils (based on FAO Luvisol classification), with an udic or ustic water regime, are more saturated in their upper horizons. High biological activity and increased mineralisational ability of the soil, in some conditions conclusively by increasing content of lower quality humus (Němeček et al., 1990), are indicated by reduced thickness of humus horizons in moderately warm and optimally wet brown soils.

The osteological assemblage was composed of the small and decaying bone and teeth fragments (several millimetres and more) with the large proportion of fractures and taphonomical changes influenced by weathering and biological processes during the deposition of the animal remains in the soil. The increased ratio of osteological assemblage fragmentarisation was undoubtedly remarkably influenced by the increased mineralisational ability and biological activity of the soil. Furthermore, diagenesis of skeletal tissues was affected by extrinsic factors, such as aeration, water regime, bacterial action, etc. The determination degree of archaeozoological assemblage was low. In the faunal list (stage of LBK of the occupation of the site Molitorov), were registered 58% of fragments of undetermined mammalian bones and 28% of fragments determined to species level. The rest of teeth and bone fragments (14%) were classified as large ruminant or small ungulate.

Archaeozoological excavated materials were washed with drinking (chlorinated) water and sequentially air-dried in the Institute of Archaeology of the Academy of Science of the Czech Republic, Prague. All dry bone remains were placed into the described paper bags at room temperature and subsequently determined. The samples were analysed 1–2 years after excavation. Osteological analysis revealed a composition of species typical of the Neolithic economy. Cattle (*Bos taurus*) dominate the collection (70%); a lesser number of bones have been determined

as ovicaprines (*Ovis*/*Capra*) – 24%. Pigs (*Sus domesticus*) were present only marginally (5%). Just one fragment of roe deer (*Capreolus capreolus*) metatarsus was recognized.

From 226 bones excavated in Molitorov locality, 28% were determined to the species level (i.e. 63 bones), 14% to the subsidiary category "large mammal" (21 bones) and "medium mammal" (13 bones). The rest (58%, i.e. 129 bones) remained in the category "undetermined mammal" (in Table 1 as a "large-medium mammal/undetermined bone"). The last category included too small bone fragments or bones without determination traits. ELISA test could thus theoretically determine 58% of bone fragments from Molitorov locality, which were not determined osteologically. In the subsidiary categories (i.e. "large mammal" or "small ungulate"), a number of bones of a particular species could be estimated on the basis of the already determined mammals. According to the osteological estimation of the small undetermined samples, cattle bones and teeth could predominate (65), followed by goats and sheep (24) and the lowest number of bones should belong to pigs (6). Such theoretical estimation could be confirmed or disproved by ELISA analysis.

The total 21 protein samples retrieved from bones were tested (Table 1). Ten samples belonged to a taxonomically determined and 11 remained undetermined; these were tested against antibodies of the three most common domesticated animals of that period: cattle, pig and sheep. We also utilised the ELISA test for the identification of the specimen origin of one outlined bone – an artefact made of incomplete diaphysis of the long bone in the shape of the bodkin (maximum length: 70 mm and width: 12 mm). Other bone samples used for ELISA test were 2–5 cm of size, but 1 mg of bone tissue is enough for one analysis. Additionally, we have tested osteologically determined samples of cattle from the Neolithic sites Chotěbudice (LBK period) and another sample of a medieval date (12th–13th centuries) from Hrdlovka. We used also recent cattle, pig, sheep, deer, and water for control of validity (Table 1). Further reference samples of cattle were obtained from various localities in the Czech Republic (Table 2).

## 4. Method

ELISA kit cooked species identification kits by Tepnel were used for detection – http://www.tepnel.com/cooked-species-identification-kits.asp. The use of these kits mentioned is referred by e.g. Björklund et al. (2001), Giovannacci et al. (2004), Hofmann et al. (1999), Pallaroni et al. (2001). The manufacturer does not mention whether these are monoclonal or polyclonal antibodies. However, it is quoted that the kit utilises antibodies to heat-stable, species-specific muscle proteins (rendered meat and bone meal). Unfortunately, the manufacturer does not mention any more specific information about target heat-stable proteins. Closer specification would be very useful for further studies.

The manufacturer says that although the antigenic protein that is used in the preparation of the test reagents is prepared from lean muscle, the tests are not muscle-specific. A wide variety of other body tissues included bone, some gelatines, blood, meat exudates/drip, egg white/yolk and cow's milk/milk products, may give positive results in the relevant test(s).

The tests are not fundamentally quantitative but rather qualitative. For this reason, we do not find it necessary to test the concentrations of proteins in the tested bones. According to the manufacturer's instruction, the trace amount of protein should suffice for the analysis. The kit is very successful in detecting animal species in food containing rendered animal meat and bone meal. The test was verified in twelve laboratories from seven European countries examining two different analytical protocols to establish the most appropriate analytical method. Meat and bone meal material was examined in this paper (Björklund et al., 2001). Statistical evaluation through applying t-statistics showed that the animal meal treated according to European legislation (> 133 °C) was significantly distinguishable from the two other test materials at a 99% confidence level using both analytical protocols. This method can be considered a complementary test to the immunoassay developed for pork detection in cooked food (Björklund et al., 2001). In our opinion, the probability is that the results obtained from ancient proteins are analogous to the results gained from the contemporary proteins because we tested denaturated proteins. The denaturation of ancient proteins itself occurs immediately before the testing.

The ELISA test differs from other common tests by the relative simplicity and easy application. We can measure about 22 samples at the same time (in the case we test 4 species) using the ELISA test. When we would like to test only one species, we can measure 40–48 samples simultaneously (we did not use this kit, but it is offered by the company). The measurements could be made by technical staff without special archaeozoological training and education. The measurements could be fully automated; a man has just to prepare samples and evaluate the results.

The danger of cross-reaction mentioned by Child and Pollard (1992) or Brandt et al. (2002) is valid at an increased ratio at ELISA reactions developed for fresh samples and consequently used for archaeological material. Nevertheless, it does not have to be true in the case of the kit we used. Detection is also focused on identification in various foods that are heat-treat prepared but the identification itself is not endangered (Björklund et al., 2001). There are also various admixtures of other epitopes and frequently in a larger amount than in the archaeological material. According to the manufacturer, the kit should even be able to limit the cross-reaction possibility, e.g. with soil bacteria (Brandt et al., 2002). Sufficient test conclusiveness should be guaranteed by focusing on heat-stable species-specific proteins with a wide representation (body tissues – including bone and also some gelatines, blood, etc.).

Beef: reacts with beef, buffalo; variable reaction to dairy products. Pork: reacts only to porcine species. Poultry: reacts with a wide variety of avian species; variable reaction to eggs.

Sheep: reacts to sheep, goat. Limit of detection inferior to 1% (beef, pork, poultry); inferior to 2% (sheep).

A small amount of bone tissue (approximately 5 mg for 5 analyses) was drilled out (using a drill of 2 mm diameter) from each examined bone fragment. The powder was

**Table 1**
The results of immune detection of bone fragment material.
**Tableau 1**
Résultats de la détection immunitaire des fragments osseux.

| | Brickworks Molitorov–Neolithic (test) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Undeter. sample | *Bos taurus* | *Sus dom.* | *Ovis aries* | Locality | Object | Dating | Archaeozoological evidence | Note |
| 1 | S1 | 0 | 0 | x | Molitorov | 15/98 | LBK | Large-medium mammal/undetermined bone, small fragment, age undetermined | |
| 2 | S2 | 0 | x | 0 | Molitorov | 8/98 | LBK-SBK | Large-medium mammal/undetermined bone, small fragment, age undetermined | |
| 3 | S3 | x | 0 | 0 | Molitorov | 30/98 | LBK | Large-medium mammal/undetermined bone, small fragment, age undetermined | |
| 4 | M1 | 0 | x | 0 | Molitorov | 30/98 | LBK | Large-medium mammal, undetermined bone, fragment, age-non juvenile | |
| 5 | M2 | x | 0 | 0 | Molitorov | 30/98 | LBK | Large-medium mammal, undetermined bone, fragment, age-non-juvenile | |
| 6 | M3 | x | 0 | 0 | Molitorov | 30/98 | LBK | Large-medium mammal, undetermined bone, fragment, age-non-juvenile | |
| 7 | M4 | ? | 0 | 0 | Molitorov | 30/98 | LBK | Large ruminant, diaphysis of long bone, fragment, age-adult | Low detection |
| 8 | M5 | 0 | 0 | x | Molitorov | 30/98 | LBK | Small ungulates, vertebra thoracica, fragment, age-adult | |
| 9 | M6 | x | 0 | 0 | Molitorov | 42/98 | LBK | Large ruminant, costa, fragment, age-adult | |
| 10 | M10 | 0 | x | 0 | Molitorov | 34/98 | LBK | Large-medium mammal, unknown vertebral fracture, age-adult | |
| 11 | M14 | 0 | x | 0 | Molitorov | 42/98 | LBK | Small ungulates, diaphysis of long bone, fragment, age-non-juvenile | |
| | Deter. sample | *Bos taurus* | *Sus dom.* | *Ovis aries* | Locality | Object | Dating | Archaeozoological evidence | Note |
| 12 | M7 | 0 | 0 | x | Molitorov | 42/98 | LBK | *Ovis*/*Capra*, metapodium, less than half of bone, age-adult | Artefact – bone bodkin |
| 13 | M8 | x | 0 | 0 | Molitorov | 42/98 | LBK | *Bos taurus*, *costa*, fragment, age-adult | |
| 14 | M9 | 0 | 0 | x | Molitorov | 34/98 | LBK | *Ovis*/*Capra*, pubis, fragment, age-adult | |
| 15 | M11 | x | 0 | 0 | Molitorov | 15/98 | LBK | *Bos taurus*, femur, fragment, age-adult | |
| 16 | M12 | x | 0 | 0 | Molitorov | 15/98 | LBK | *Bos taurus*, femur, fragment, age-adult | |
| 17 | M13 | x | 0 | 0 | Molitorov | 42/98 | LBK | *Bos taurus*, frontal, less than half of bone, age-adult | Maybe higher level of aurochs' protein |
| 18 | M15 | x | 0 | 0 | Molitorov | 42/98 | LBK | *Bos taurus*, radius, proximal part, less than half of bone, age-adult | Scorch |
| 19 | M16 | 0 | 0 | x | Molitorov | 42/98 | LBK | *Ovis aries*, scapula, distal part, half of bone, age-adult | |
| 20 | M17 | x | 0 | 0 | Molitorov | 38/98 | LBK | *Bos taurus*, humerus, distal part, half of bone, age-subadult-adult | Burnt bone |
| 21 | M18 | x | 0 | 0 | Molitorov | 42/98 | LBK | *Bos taurus*, frontal, less than half of bone, age-adult | Maybe higher level of aurochs' protein |
| | Control | *Bos taurus* | *Sus dom.* | *Ovis aries* | | | | | |
| Bos | | x | 0 | 0 | | | | | KIT Components |
| Sus | | 0 | x | 0 | | | | | KIT Components |
| Ovis | | 0 | 0 | x | | | | | KIT Components |
| Bos | | x | 0 | 0 | | | | | Recent sample |
| Sus | | 0 | x | 0 | | | | | Recent sample |
| Ovis | | 0 | 0 | x | | | | | Recent sample |
| Cervus | | 0 | 0 | 0 | Denmark | | Chalcolithic | | |
| Water | | 0 | 0 | 0 | | | | | |
| Water | | 0 | 0 | 0 | | | | | |
| water | | 0 | 0 | 0 | | | | | |

Notes: x: positive reaction; 0: negative reaction; ?: low reaction; LBK: Neolithic (Linear Pottery culture); SBK: Neolithic (Stichbanderkeramik).

**Table 2**
The testing of intensity immune reaction of cattle.
**Tableau 2**
Intensité de la réaction immunitaire chez les bovins.

| | *Bos taurus* | *Sus dom.* | *Ovis aries* | Locality | Object | Dating |
|---|---|---|---|---|---|---|
| *Aurochs – Bos primigenius* | x | 0 | 0 | Staré Badry | 88 | Early Middle Ages |
| *Aurochs – Bos primigenius* | x | 0 | 0 | Ústí nad Labem | | Middle Ages |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Chotěbudice | 41 | LBK |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Hrdlovka | P 61 | 12–13 cent. |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Mělník-Česká | | Middle Ages |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Cheb-Dlouhá 21 | S1 | Middle Ages |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Velká Chuchle | 42 | Chalcolithic |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Dubeč | 706 | Bronze Age/Iron Age |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Písek | | Middle Ages |
| *Bos primigenius* f. *taurus* | x | 0 | 0 | Liba | | Middle Ages |
| 5 times recent samples | x | 0 | 0 | | | |
| Positive control | x | 0 | 0 | | | |
| Negative control | 0 | 0 | 0 | | | |

obtained in this way from every sample (Fig. 2). All the samples were then boiled in salted (9 g/l) distilled water (0.9% physiological solution) at 100 °C for 15 minutes, placed in microcentrifuge tubes (Eppendorf) whose caps were to be perforated to prevent opening from inner gas pressure. This way of treating samples was chosen to reach maximum agreement with the instructions of the producer, who recommends the preparation mentioned above in the case of unboiled samples. According to the manufacturer, the target proteins should be unaffected due to their heat stability. All the measurements were repeated two times in our different laboratories for control.

Solutions created by this procedure were placed into a microwell module (Fig. 3), so that three 100 μl subsamples of each sample went in respective microwells provided with species-specific protein antibodies (beef, pork and sheep). Fresh samples taken from bones and meat of recent animals were examined to check this test. They were, as well as all the samples, boiled in 0.9% physiological solution.

The next course of the analysis corresponded with the instructions of the ELISA test manufacturer included in the kit manual (Tepnel company BioKits – cooked species identification test kit).

The cooked species identification kits are sandwich-type EIAs utilising a biotin-avidin enhancement process. During this procedure, specific proteins from a sample extract binds on the primary antibody (species-specific) at the bottom of the wells (antibody-coated). Samples are extracted using a simple saline solution. A positive control was prepared by adding 100 μl of kit controls/extracts for each species (beef, pork and sheep) for visual comparison with tested samples. Similarly, 100 μl of distilled water was used as a negative control. The incubation of sample proteins with a biotinylated species antibody locked in each microwell took 45 minutes. After this period, all solutions were removed from wells, which were immediately washed three times with the working wash solution (part of the ELISA kit). Next, 50 μl of anti-species biotinylate (secondary antibody) was added, respectively, for each tested species (beef, pork and sheep) and incubated for 45 minutes. Then, the microwells were washed three times. Subsequently, 50 μl of avidin peroxidase conjugate was added and the incubation lasted 15 minutes. After this step,

**Fig. 2.** Taking a sample from a fragment bone.
**Fig. 2.** Échantillonage de tissu osseux.

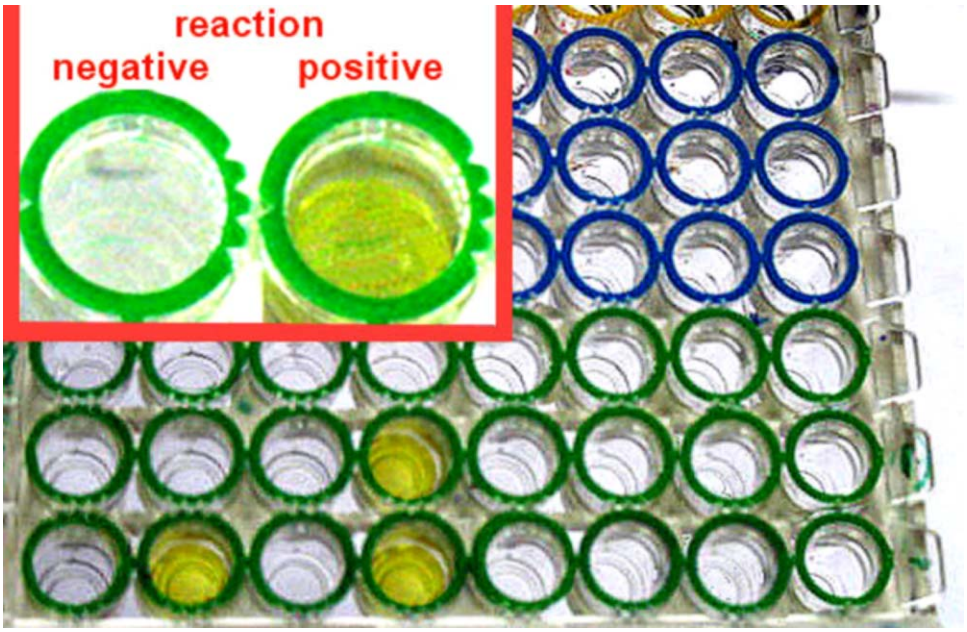


**Fig. 3.** The microwells with positive and negative reactions (yellow – positive reaction; negative – clear).
**Fig. 3.** Lecteur plaque microtitre avec réaction positive et négative (jaune – réaction positive ; claire – réaction négative).

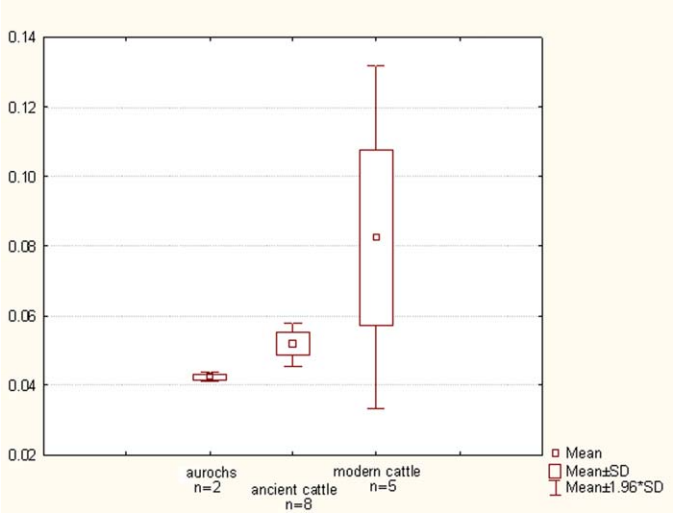


**Fig. 4.** The preliminary comparison of absorbance values of aurochs ($n = 2$), ancient cattle ($n = 8$) and recent ($n = 5$) samples analysed by Elisa test. Measurement wavelength was 450 nm.
**Fig. 4.** Comparaison préliminaire des mesures de l'absorbance chez les aurochs ($n = 2$), bovins actuels ($n = 5$) et anciens ($n = 8$) par le test immunologique Elisa. Valeur moyenne de l'absorbance iA = 450 nm.

washing had to be performed five times. In the last step, we put 100 μl of TMB Solution into each tested microwell for a further 45 minutes of incubation (bound peroxidase activity is determined by adding a fixed amount of TMB substrate which develops a blue colour in the presence of peroxidase, changing to yellow after the addition of acid stop reagent). The reactions were stopped by adding 50 μl of commercial Stop solution. After ten minutes, the reaction results were observed. A qualitative interpretation can be easily obtained by comparison against the positive and negative kit controls. The positive reaction is signalled by the yellow colour of the final product, while the negative one remains clear (Fig. 3). Detection was done mostly by sight; some results were compared with the aid of a spectrophotometer (BOECO Uvi Light XS), measuring 450 nm, against the negative control (Fig. 4). During spectrophotometric measurement, obtained values were compared with water samples.

## 5. Results

We tested if the kit could correctly detect the skeletal material and we applied this analysis to the recent

and well-preserved medieval bones, which were reliably morphologically determined. In the Molitorov set of 21 samples, cattle were identified twelve times, pig four times and sheep five times (Table 1). Two animal bones were less typical. The first bone served as an archaeological artefact (M7) and the second bone was burnt (M17). Both "atypical" bones showed a standard reaction with the antibody in the analysis. As regards the artefact, it was proved than it had been made from sheep bone. The burnt fragment belonged to cattle.

The analytical results corresponded unambiguously with the morphological findings. Among small undetermined bone fragments, the ELISA test revealed sheep, pig and cattle; the latter comprised nearly half of the undetermined samples. Two samples were determined osteologically as small ungulates, and the ELISA test identified sheep and pig. Two samples determined as large ruminant showed reaction typical for cattle. Cattle bones could predominate in the material, followed by goats or sheep and pigs as the rarest species, according to the osteological estimation of the undetermined bone fragments. In comparison to the osteological estimation, the ELISA tests indicated the same proportion of pig and cattle in the material, but only two sheep.

We examined a possibility that the kit gives a negative reaction with wild species, i.e. cervids that can be found in the archaeological sites from the same period. Chalcolithic deer sample gave negative results. Fresh control meat and bone samples boiled for a while showed a positive reaction similar to the one in the control kit. This was remarkable especially in the case of cattle when a weaker reaction of Neolithic samples was shown.

During the first series of experiments, there, appeared in two cases a weak positive reaction to other species besides the one dominant positive reaction. This problem was probably caused by cross-contamination between samples during the microwells washing. In the second series of experiments, no cross-contaminations appeared. This is a promising result; future experiments should nevertheless take into account possible means of sample contamination and try to identify whether the weak reactions sometimes occurring beside a strong one represent a contamination introduced by natural processes in an archaeological deposit or later during laboratory treatment. Neither of these can be ruled out at the moment.

Unexpectedly, a positive reaction for cattle was always remarkably weaker than in the case of positive reactions for pig and sheep, but still easily distinguishable from the negative control. In one unusual case (sample M13), the result seemed to be closer to negative than to positive control; nevertheless, a repeated test with another part of the same bone showed a reaction characteristic rather for cattle. Bone samples which have been determined according to their morphological traits as belonging to cattle were used as control samples for evaluation of the method and also for comparison with aurochs (Table 2, Fig. 4).

Cattle samples in Table 2 originated from different localities and time periods, but all of them were found in the Czech Republic. This was intended as an experiment to test whether the unusual (weak) positive reaction is only a matter of the prehistoric period (LBK, Chalcolithic, Bronze age/Iron age) or whether it gives the same result also for the samples dated to the Middle Ages. The latter was the case. No observable difference was noticed among the weaker positive reactions produced by samples from various past time periods. We supposed genetically different populations – aurochs, ancient + Middle Age and recent cattle. Results of ELISA tests measured spectrophotometrically revealed possible differences among these populations (Fig. 4). Tests of determined cattle bones demonstrated that the kit possibly could differentiate samples of various cattle populations. However, small number of examined samples, especially in the case of aurochs, makes this observation only preliminary and uncertain.

## 6. Discussion

The results achieved so far suggest that it is possible to determine the species of domestic animals from highly fragmented bone material using the ELISA method. Moreover, this approach seems to have some advantages over detection based on aDNA, at least in some aspects. The aDNA indeed remains the most important source of genetic information; in our study, we just tried to find easier and cheaper alternative to some routine analyses. Our test focused on proteins denaturated by heat. Denaturation eliminates differences between recent and ancient proteins. This is the reason why this kit for detection of cooked food is more suitable for detection of ancient proteins than antibodies used earlier.

A very small amount of bone mass (1 mg) usually suffices for the analysis and the method is therefore less destructive than aDNA analyses. The method is also faster and shows a higher rate of results achieved. Furthermore, we have observed a lower cross-reaction rate than Reuther et al. (2006) who tested pRIA method for a similar task. Due to the repeated experiments, we concluded that in our case, did not occur cross-reactions but cross-contaminations. Better knowledge of experimental routine prevented cross-contamination in the second series of experiments. Some cross-reactions could be caused by humic acids co-extracted during the procedure, which specifically bind into the ELISA wells to antibodies raised against different taxons.

Species determination using collagen peptide (Buckley et al., 2008) is another promising method in archaeozoology, besides the method MALDI, which has already became relatively common. However, we think that immunodetection could be sufficient in common routine; especially for more extensive analyses, because it is quick and does not require specialized technical equipment. For elementary determination optical detection is adequate.

Unfortunately, according to the manufacturer, the kit can determine goat protein as sheep. However, our preliminary results show that there is some possibility of distinction (data not shown). We would like further examine its ability to distinguish sheep and goat bones.

Original osteological analysis of the bone collection from the Molitorov Neolithic settlement successfully determined only 28% of the total number of specimens. Moreover, we can assume a bias towards more robust and compact bones and teeth in this number because

these osteological elements have been more resistant towards the impact of the environmental influence. Larger bones usually display more determination markers than smaller ones and are easier to determine. We supposed that osteological analysis could partly marginalized the small ungulates due to an increasing fragmentation of their bones in consequence of the taphonomic processes (Lyman, 1994). Results of ELISA test revealed slight predominance of small ungulates, but we cannot consider smaller species to be markedly underestimated due to the increased fragmentation of their bone remains. Analysis of a larger sample size could clarify the situation.

ELISA tests confirmed and specified results of osteological determination of the smaller bone fragments to either large-medium mammal category (i.e. cattle in ELISA analyses) or small ungulate category (i.e. pig, sheep or goat in ELISA analyses). According to the osteological estimation of the small undetermined samples, cattle bones could predominate, followed by goats and sheep and the smallest number of bones should belong to pigs. In comparison to the osteological estimation, that has particularly stressed the consumption of cattle, the ELISA tests indicated the same proportion of pig and cattle in the material and only two sheep. The results of ELISA analyses are congruent with taphonomical predictions. Pig remains could probably preserve better than sheep or goat (Ioannidou, 2003; Lyman, 1994).

Our result shows slight differences among ancient (Middle Age) cattle, recent cattle and auroch samples. The strongest reaction shows recent samples, weaker reaction is characteristic for ancient and Middle Age bones and the weakest reaction displays aurochs, despite the fact they were dated to the Middle Ages. Our results possibly indicate differences among various populations and perhaps some introduction of aurochs to the domesticated population on Czech territory. Aurochs' influence could be eliminated by modern selection. However, we had only a few samples for analysis available, in the case of aurochs, there were only two reliably determined bones. Therefore, the total number of measurements was not representative.

The question as to why there was a weak reaction with cattle antibody can be hypothetically explained by the genetic variability within and between ancient populations of domesticated animals. It could be caused by many different causes, including small immunological differences between the ancient and modern lineages due to very recent zootechnical improvements of the modern breeds (pers. com. anonymous reviewer). Our ancient Czech sample could have had a much stronger affiliation to aurochs than modern cattle, unlike the rest of the tested specimens with a positive but still weaker response. However, it is still unclear. Introduction of aurochs to the breed originally domesticated in Near East was not as important as it seemed (Götherström et al., 2005). Geographical distribution of haplogroups examined using intron of the Y-chromosomal gene *UTY19* was not confirmed too (Svensson and Götherström, 2008). Nevertheless, in our opinion, complete denying of aurochs males' introduction on domestic populations is not reasonable for the present. Contradiction of this hypothesis just on the basis of mitochondrial DNA analyses is premature (Bollongino et al., 2006, 2008a; Edwards et al., 2004, 2007; Stock et al., 2009). The introduction of aurochs males and not females is usually assumed. The results based on the distribution of Y chromosome could be influenced by the relatively small number of analysed samples (Svensson and Götherström (2008) or Bollongino et al. (2008a) who analysed only 13 ancient males). On the other hand, there are 180 analysed samples from recent populations (Götherström et al., 2005). These data are difficult to compare reasonably.

Aurochs problematic could be clarified by careful analysis of some selected proteins. Our study represents an initial step on this way. Laboratory testing of animal bone taxonomic affiliation based on proteins can become a valuable alternative to aDNA research. The method used in our case study is faster and less cumbersome than DNA extraction, amplification and other related techniques necessary for DNA sample processing. The probability of protein preservation in archaeological material is also higher than that of aDNA. Using the ELISA kits is aimed at proteins that were treated by heat. The analytical approach offers a given potential for the successful determination of even bones which have been singed or burned. The testing of modified bones and used as artefacts and therefore not easily determined by traditional osteological methods is another important direction of this kind of research. The relative simplicity and promptness of the ELISA test application is a virtue especially appreciated in archaeology because a sufficient laboratory can be run even in adverse field conditions.

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