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COLOSTRUM A NATURAL VALUABLE PRODUCT FOR NEW-BORN ANIMAL AND INDUSTRY

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Summary

Farmers and food industry are very interesting of the health status of animals and quality of animal product. In this context, colostrum through its composition is a valuable product, assuring the nutrients for the new-born animal. Colostrum and milk composition can be also be used to evaluate the health status for mother and baby animal, because through these products the nutrients and possible pathogen agents can be transfer from mother to the baby. But mammalian colostrum is also a product of supplements industry, being rich in proteins (such as: immunoglobulins, lactoferrin, haptoglobin, osteopontin, amyloid A, gelsolin), cytokines and amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine and tyrosine), vitamins (tocopherols, retinols, ascorbic acid, B6 and B12), fatty acids (essential polyunsaturated fatty acids), minerals (calcium, potassium, iron, phosphorus, zinc, chromium), growth factors (GF), antimicrobial factors, carbohydrates and lipids. In industry, the colostrum (most bovine colostrum) is transformed in powder supplements for humans and animals. Research data proved that colostrum supports healthy digestion, increases immune system, improves athletic performance and recovery time, increases lean muscle mass, and decreasing the allergy symptoms. Industrially the colostrum can be preserved as micronized, micro-encapsulated, instantized and lyophilized product.

Key words: colostrum, animals, industry

After birth, the new-born animal has to adapt to all environment and nutritional conditions. Most of the time coming from a sterile environment in a stable, the neonate has to find immunological solutions to survive in a good health condition. Also, the new-born animal is ready to walk and follow its mother in just few hours after birth, which involves special energetic needs, and continuous changes of metabolic status. And all these biochemistry mechanisms are possible due to the nutrients found only in the colostrum – the only natural, complete and unmatched feed plan for the infant animal. However, studies demonstrated that fresh colostrum is more efficient on development and on innate immunity function in mammals (bovine, goat, sheep, equine, and not only),
but overall the colostrum administration on infants ensure adequate passive immunity transfer (4, 11, 14, 21, 38).

Colostrum benefits were known since ancient, this “food” being mentioned in the work written of 300-275 BC, entitled: “The Book of Sirach”, where “the first milk” was ranked alongside water, wheat, salt, honey, fire, and iron (The Apocrypha and Pseudepigrapha of The Old Testament, by R.H. Charles – cited by Oxford University Press, 1976) (49). Between 1950-1990 years the researches in pharmaceutical field provided synthetic alternative of colostrum. But, because time proved the natural, intact, superior benefits of the colostrum, lately, the medicine and nutrition encourages consumption and use the colostrum administration as a feed plan for infants, and also as a natural supplement for animals and humans (40).

In Egyptians ancient images, hieroglyphic texts presented the goddess suckling the Pharaoh, symbolizing the colostrum as an immortality elixir for the king. Also, in India the cows are protected and consider sacred animal, and the colostrum was being used by Ayurvedic physicians in skin and eye treatment for various conditions. In Sweden, The Netherlands and England, colostrum is a folk cure and tonic, being known also as “beesting”. In Kenya the tribesmen drink bovine colostrum to enhance their vitality and strength (40).

In 1962, Dr. Albert B. Sabin and Dr. Howard Fieldsteel tested the efficiency of colostrum and late milk against the Lansing strain of poliovirus (type 2) in mice. They found out antipoliomyelitic activity in most of the samples, but only when the antibody was also present in the serum. Their research demonstrated that when the milk was sampled from the mothers tested and confirmed with serum poliovirus (type 2) antibody, the rate of virus neutralization was 85% of 25 mothers tested in the days 2 to 6 days after parturition, and 62% of the 39 mothers tested in the days 35-356 days after parturition. Also, they proved that the concentration of antipoliomyelitic compound was higher in colostrum (1:26 versus 50LD$_{50}$ of virus, as average titer) compared to the mature milk (1:5 versus 50LD$_{50}$ of virus, as average titer) (41).

Starting with these ideas, the researchers analyze the colostrum composition for independent animals (as wild animals) or captivity animals (for farm animals or for captive wild animals considered animals for breed conservation). Knowing these, the animals were fed ad libitum only with colostrum, and usually their energy requirements are not essentially, but it is very important the nutrients quality of the colostrum. However, the nutritional composition of colostrum is very important in animal sciences for several situations, such as: increasing the survival rate for breeding conservation, treating various metabolic diseases, helping the infants in special conditions (when infants are born in adverse environmental conditions, mother is ill or died, and other situations) (46). Also, the scientists test the colostrum composition and try to find the best solution for colostrum preservation.
Passive immunity and health enhancement from colostrum

Maternal ready-made antibodies can be transferred to the fetus (across placenta) or to the infants (through colostrum). Passive immunity is defined as a special antigen resistance that can be achieved naturally (placenta or breastfeeding in mammals, or during hatching in birds) or artificially (by supplementation with immunized serum or colostrum administration) (26, 27). The passive immunity is a process in which the immunoglobulin can be transferred in the third semester of pregnancy, by the Fc-Rn receptor from placental cells.

The passive immunization of the mammalian fetus and neonates is possible due to endocytosis of immunoglobulins by mucosal cells and then are exocytosed into the intestinal lymph. In primates, the immunoglobulins are transferred to the fetus across the placenta since the fetal stage, being considering a passive immunization with high impact on the neonates’ health (19).

Immunoglobulins in colostrum constitute about half of the proteins. These colostrum immunoglobulins are absorbed intact in a very short period of time after neonate ingestion (24-36 hours after parturition), but these can also be digested by mucosal cells. In neonates the intraluminal protein can't be complete digested because into the stomach it is not produced enough hydrochloric acid (HCl) necessary to inactivate the pepsinogen. Also, in colostrum is found a tripsin inhibitor that prevents the luminal activation of the trypsin.

Approximately 75% of total immunoglobulins in adults are represented by IgG, being high concentrated in plasma, but it is found in all extracellular fluids. IgG – through aggregation form proteins with soluble antigenic has properties which enhanced the phagocytosis (by reticuloendothelial system). During the pregnancy (between weeks 18-20) the fetus is humoral immunized with IgG which is efficacious transported across placenta (7). Thus, immunoglobulins (IgA and IgG) protects the fetus and infant against bacterial or viral infections, preventing various possible diseases (tuberculosis, hepatitis – especially hepatitis B, poliomyelitis, pertussis, enteritis, and others) (17, 18, 39, 42). Structurally IgA is similar to IgG. IgA is found usually in monocovalent association with a highly glycosylated peptide – which is synthetized by mucosal cells, because the peptide protects the IgA against proteolytic digestion. Also, IgA can be found in dimerized form in secretions of parotid, intestinal and bronchia (7). Some of the researchers consider the colostrum as a protective vaccine for the infant first year of life (29).

Lately, the breastfeeding in humans is consider the most healthy nutrition way for infants, providing proper development and growth requirements for neonates and also for mother. The colostrum components compared to mature milk assure immunologic factors, acting as a health protection mediator (23).
Colostrum composition

The composition of colostrum depends very much by animal species, breed, parturition, age, heath status, environmental conditions, and maybe the most important aspect is the time of sampling the colostrum after parturition.

Because researchers are overview both terrestrial and aquatic mammals, not only the common animals were subjected to colostrum composition research, but also odontocete (dolphins and whales) were under attention of biologists.

Protein, hormones and enzymes

The most important and valuable component of the colostrum, no matter from what animal species, are the proteins. Passive immunization in mammals is possible during the pregnancy – from mother to the fetus – across placenta and right after parturition – across the colostrum feeding, and latter through the breast-milk feeding. The bioactive components of colostrum act against pathogen agents, due to the presence of immunoglobulines, insulin-like growth factors (I and II), lactoferrin, lactoperoxidase (the major antibacterial enzyme in colostrum), lysozyme (an enzyme with antibacterial and lytic properties) and cytokines, making possible the passive immunization (23, 32, 43) – see more details in Passive Immunity from colostrum chapter.

In vivo and in vitro tests proved the preventive and treatment potential of colostrum and milk due to their composition in peptides and proteins. Thus, the casein, casein peptides and casein hydrolysates have hypotensive action, reduced the tumor cell proliferation rate, act as protective against diabetes, and presented antioxidant, anticoagulant, antibacterial and antiviral properties. Formulas based on lysosome supplementation in infants, present anti-inflammatory and analgesic effect in neoplastic diseases. Also, α-lactalbumin and β-lactoglobulin used as diet supplement demonstrate an HIV-antiviral, antibacterial, antistress and anti-depressive, anticarcinogenic activities, and decrease the blood pressure. Colostrum lactoferrin manifests antimicrobial activity (bacterial, viral and fungal), antiparasitic and antithrombotic effect. As well, colostrinin (a protein rich in proline) proved good results in experiments of neurodegenerative and autoimmune diseases (13, 39).

The leukocytes present in colostrum are very important for newborn infant and also for the mother. Thus, the leukocytes protect the mother mammary gland against infections during lactation, and because of this, if the breast is not empty regularly, the ducts will become blocked, the microbes will migrate along the mammary ducts and the mammary gland will inflame resulting mastitis, abscess, and even septicemia unless immediate and appropriate measures are applied. But organism is notified in such cases, and even in mild nipple injuries, the organism increased the leukocytes influx to help healing (7).

New researches try to demonstrate which exactly the responsible components are for classify the colostrum as an unmatched food. Thereby, recent published research brings in our attention the fact that it could be possible that only some proteins like: haptoglobin, osteopontin, milk amyloid A, gelsolin, can make the difference between the bovine colostrum and bovine mature milk. This study
made a comprehensive comparison of colostrum proteome and mature milk proteome (in rank and ratio) (34). Thus, in cellular fractions of bovine colostrum were detected 50 different proteins presented only in colostrum, 13 different proteins presented exclusively in mature milk, and 99 different proteins common presented both in bovine colostrum and milk. Having this new laboratory information, it is possible that the specific proteins of colostrum to make the functional difference between colostrum and mature milk (34). Also, Amyloid A3 protein (noted as AA3 too, has protective role in neonatal intestine) in equine colostrum and in early milk is not effecting by age and breed, but increased gestation length and parturition induction decreases its concentration (16).

Bioactive role of proteins presented in swine colostrum are protected against the gastric proteolytic degradation, and then are rapidly internalized and retained in the cells from small intestine tissue – fact that proves the proteins' functional activity (12).

The colostrum of *Alxa Bactrian* camel is also rich in immunoglobulin and albumins proteins, but the concentration is reduced from the second day of lactation (45).

The main present enzymes in colostrum are represented by $\alpha$-amylase, protease, protease inhibitor, and lactoperoxydase (3).

Oxytocin is also known as “feeling good” hormone, and recent research on pigs demonstrated that maternal ability of sows is linked to the oxytocin releasing depended by physical contact between sows and piglets. Thus, oxytocin synthesis occurs in neuronal brain cells and in specific tissues involved in reproduction mechanism, being a necessary hormone in parturition and lactation, suppressing the stress and anxiety (20).

*Lipids and fatty acids in colostrum*

Many researches were performed on colostrum and milk composition for different animals' species. In Northern Ireland a number of 1,239 samples of bovine colostrum were analyzed for lipids, proteins, lactose and immunoglobulin concentration. So, in bovine colostrum the authors detected 6.4% total fat, 14% total proteins, 2.7% lactose, and 55mg/mL IgG (18).

Thus, Marounek and his collaborators (2012) tested how the goat colostrum and milk fatty acids composition is changed during the first month after parturition [33]. Thereby, their research established that the saturated fatty acids from total taffy acids decrease from 67.0% in colostrum to 62.0% in the 30th day after parturition. From total fatty acids, the monounsaturated fatty acids in colostrum were 28.2% and the concentration increased progressive with the lactation. Also, the polyunsaturated fatty acids was higher in milk (4.8%) compared to colostrum (4.4%). Composition in fatty acids varied during the lactation, the concentration of some fatty acids increases and for others decreases. Thus, the highest concentration of fatty acid in goat colostrum and milk was registered for palmitic acid (30.1% in colostrum and 23.6% in milk), followed by oleic acid (25.3% and 30.3%), then stearic acid (11.8% and 13.6%), and miristic acid (11.4% and 8.6%). Caprylic, capric and lauric acids (medium-chain fatty acids) also presented
increasing concentration in goat colostrum (8.7%) compared to mature milk (11.1%). Polyunsaturated fatty acids are very important also for immunity, being efficient as antimicrobials, protecting the new-born and infant goat from microbial pathogens (33).

Mares’ colostrum comparing with mature milk was also tested in the first and second day after parturition and at every 4 weeks, starting with the first month of lactation. The total lipids, fatty acids percentage rate and cholesterol presented statistically significant variations between colostrum and milk. Polyenoic fatty acids (n-6 and n-3 family) ratio was significant correlated with the lactation period, presented important concentration changes (37).

Most of the mammalian have higher lipid concentration in colostrum compared to mature milk, but camel colostrum is lower in total lipids compared to milk (45).

Also, in captive bottlenose dolphin the total lipids and crude protein increase significantly in the first 3 months of lactation (47).

**Carbohydrates in colostrum**

The average lactose content of camel colostrum is 4.44% at two hours after parturition, and in mature milk (in the 90th day after parturition) decreases slightly to 4.24% (45).

The colostrum composition was not analyzed only in terrestrial mammals, but also in aquatic mammals. Thus, some Japanese researchers analyzed the composition in trisaccharide and tetrasaccharide of bottlenose dolphin colostrum. These carbohydrates (oligosaccharides) were not previously found in any other natural sources, not even in colostrum or milk, but were detected as components of GM2 and Gb3 glycosphingolipids (44). As well, in captive *Tursiops truncatus* (bottlenose dolphin) the sugar content was not modified in the first 3 months of lactation, but the energy percent provided by crude protein decreases significant, but slightly (47).

**Vitamins and minerals in colostrum**

Bovine and goat colostrum presented higher concentration of copper, manganese, iron and zinc in colostrum sampled at 6th hours, compared to 24hours, and 48 hours after parturition. Ewe colostrum presented higher content of copper, manganese and zinc, but iron increased in the first 6 hours of lactation compared to 24 and 48 hours of lactation (3).

Calcium and phosphorus content in camel (*Alxa Bactrian*) colostrum decreased in mature milk (90th day after parturition), but sodium, potassium and chlorine increased in mature milk compared to colostrum (2 hours after parturition). Camel colostrum contains vitamin A, E, D, thiamine, riboflavin, pyridoxine, and ascorbic acid. The tocopherol and thiamine content was higher in camel colostrum and retinols and ascorbic acid were lower in colostrum compared to mature milk (45).
Colostrum as a protective and treatment product

Due to the proteins and peptides presented in colostrum and milk in the first month after parturition these nutraceutical valuable natural products can be used in prevention or treatment of some diseases, both in animals and humans. Some experimental researches demonstrated that peptides and proteins from colostrum have antibacterial, antiviral, fungistatic, antiparasitic, antioxidant and anticoagulant activity; reduces the tumor cell proliferation, have hypotensive action, act in diabetic prevention, present antistress, antidepressive, antithrombotic, anti-inflammatory and analgesic activity (13).

Administration of colostrum to athletic people demonstrated that boosted the immune system and helped in sickness recovery (8, 10, 24). Also, the colostrum supplements improve the growth and development of lean muscle tissue (especially when the administration is associated with creatine and whey protein) (28). It is a very good natural supplement for athletic women because its administration did not increase the testosterone level. Also, due to its composition in Tumor factor-alpha (noted as TNF-a), the colostrum reduced pain in inflammation or swelling. Another very important protein found in colostrum interleukin-1ra (IL-1ra) is reduces the pain, swelling and inflammation. Other peptides from colostrum, like proline rich peptides (PRPs), protect the organism against oxidative stress.

Another protective action of colostrum is to reduce the synthesis of immunoglobulin E (IgE) – an immunoprotein expressed by organism in case of allergy or in case of asthmatic attack (when a foreign protein enter into the organism and produce an allergic reaction).

Colostrum is used as protective and improving the treatment in cognitive disorders, especially Alzheimer's disease – acting as a product that reduce the severity of the disease. Researchers presented the magical component acting in this direction being represented by phosphatilylserine (Ptd-L-Ser or PS) – a phospholipid present in the membrane of the cell, with important role in cell cycle signaling, especially in apoptosis.

The presence of glutathione in colostrum proved the antioxidant effect and the action in hepatic tissue detoxification.

Also, genetic engineering techniques applied in animal breeding (15) can lead to animals that produce colostrum with a higher concentration of immunoglobulin and other bioactive components in colostrum, the maternal instinct is emphasized – and this can be considered an efficient method for diseases prevention, both in animals and human (22).

Preservation of the colostrum

There are several methods to preserving colostrum, such as: micronized, micro-encapsulated, instantized and lyophilized (25, 30, 35, 36, 48). Preserved...
colostrum has already found many uses in the medical and pharmaceutical industries, in animal husbandry, and also in food technology (1, 2, 6, 9).

Encapsulation process can be performed by spary drying, extrusion, molecular inclusion in cyclodextrins, coacervation, centrifugal extrusion, air suspension coating, spray chilling and spray cooling, centrifugal suspension-separation, freeze-drying, and co-crystallization. For micro-encapsulation it is very important to establish the method and all the materials and ingredients, thus the coating material for encapsulation has to be chosen depending on the types of encapsulated food ingredients, and last but not the least the microcapsules has to be analyzed for their final structure and the releasing mechanism (5).

Another preservation method, large used in pharmacological industry, is liposomal colostrum. The liposome is a vesicle, spherical, with at least one lipid bilayer, usually phospholipids (phosphatidylcholine or phosphatidylethanolamine from egg). Liposomes can be obtained by disruption of biological membrane by different methods, such as sonication. The liposomes can present surface ligands to assure the possibility to bind different ligands. According to Liu and his collaborators (2012), milk fat globule phospholipid membrane can be used to liposome preparation for different bioactive components using the method of thin film ultrasonic dispersion and also by dynamic, high pressure, micro-fluidization method (31).

Conclusion

Colostrum is natural and valuable product secreted and eliminated by mammals in the first hours after parturition. The composition and quantity of colostrum depends very much by various factors, such as: mammal species (terrestrial or aquatic), mammal breed and other genetic characteristics, health condition of the mother, nutritional and environmental conditions.

Composition of colostrum also varies very much if the animals are independent (wild animals) or in captivity (farm animals or captive animals for breed conservation).

Befits of colostrum administration were demonstrated along centuries, and first proves were presented as hieroglyphs or images since antiquity. Now a day, the researches observations and results presents very clear the efficiency of colostrum administration for mother, neonates, industry and economy.

Lately, the preservation methods for colostrum include modern techniques, such as: micro-encapsulated, micronized, instantized, lyophilization or liposomal encapsulation of colostrum. The preserving colostrum possibilities help surviving the poor condition of neonates, breeding conservation, keeping good health condition, recovery in athletes, prevention and treatment of some diseases, anti-aging, and provide the possibility of using the colostrum anywhere in the world and anytime when needed.
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ANALYSIS OF GENETIC POLYMORPHISM IN DOG BREEDS
USING MOLECULAR MARKERS SYSTEMS

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Summary
Molecular markers have an important role in estimating the genetic diversity of same specie individuals by comparing their genetic fingerprint at a number of polymorphic loci. The effectiveness of polymorphic DNA markers in phylogeny testing and breed identification has been proven in many species, including dogs but this technique has not been used to control for pedigrees in large populations of related races. Dog breeds were created by rigorous selection of phenotypic traits and this resulted in changes in genetic information. The present study is presenting a genetic diversity evaluation of 14 dog breeds using the PCR technique and three molecular markers systems, DAMD (directed amplified mini satellite DNA), ISSR (inter simple sequence repeat) and SCot (Start codon targeted). Scoring the obtained molecular data a common binary matrix was developed and, applying Jaccard coefficient the genetic distances and similarity indices were calculated. Based on that data set, a dendrogram containing the phylogenetic relationship among dog breeds was generated. The result revealed that the used molecular markers systems were useful in describing genetic variation and similarities within breeds and moreover, establishing the genetic relatedness between breeds, the method emerging as a potential candidate in identifying the appurtenance to a pure breed process.

Key words: dog breed, molecular markers, genetic similarity, genetic divergence

The domestic dogs stem from a common ancestral group some 15–30,000 years ago and have since spread all over the world (12, 14, 15), continuously adapting to human environments by using the same shelters and food resources. Nowadays, purebred domestic dogs exist in small homogeneous strains called breeds. These breeds were created through backcrossing and inbreeding, resulting in a reduced level of heterozygosity those strains when compared to wild ascendants (10). Based on available breed histories, the majority of more than 400 dog breeds were developed in the 19th century. The new dog breeds were developed from existing breeds and are established by a particular phenotypic trait. Polymorphic molecular markers have been used to study genetic diversity in several dog breeds, mainly for the parentage applications, match probability for forensic casework, and characterization prior to linkage analysis in specific breeds.
In addition, the phylogeny in three and five breeds, respectively, has been investigated using sets of polymorphic VNTR markers systems. Neutral genetic markers are an important tool for scoring genetic diversity and the similarity of living species (6), because the genomes of eukaryotes can be divided into different classes on the basis of functionality: the so-coding regions that contain DNA sequences (genes), and the non-coding DNA that contains sequences for which no function has yet been established. Such sequences may either be single copy (‘spacer DNA’ between the coding regions of the genome) or exist in multiple copies, thus being called repetitive DNA. 20 to 30 per cent of the genome is comprised of repetitive sequences, these having no clearly ascribed functional attributes. The centers and tops (telomeres) of chromosomes are constructed predominantly of tandem repeats. The polymorphisms arising by described mechanism have been referred to as ‘mini and microsatellites or variable number of tandem repeats (VNTRS) (11). VNTR markers are dispersed throughout the eukaryotic nuclear genome and their polymorphisms are the result of variations in the number of tandem repeats in a short core sequence (5). VNTR markers have two main classes – microsatellites and minisatellites – both being characterized by a high degree of length polymorphism. Microsatellites are tandemly repeated motifs of 1–6 bases and can repeat from about 5–100 times at each locus. Minisatellites are tandemly repeating motifs of 8–100 bases that can repeat from two to several hundred times at each locus. Microsatellites are more or less randomly scattered throughout the genome and frequently appear in transcription units. In contrast, minisatellites are interspersed but often clustered in telomeric regions. In this study the two described VNTR markers systems techniques were used: Inter simple sequence repeat markers (ISSR) (16) - primers based on microsatellites are utilized to amplify inter-SSR DNA sequences, and Direct amplification of minisatellite DNA markers (DAMD-PCR), (8), has been explored as a means of generating DNA probes useful for detecting polymorphism. Those markers can yield individual-specific DNA fingerprinting pattern and thus have the potential as markers for species differentiation and breed identification. The genetic diversity can also be successfully scored using Start Codon Targeted (ScoT) molecular markers technique this being a DNA marker system based on conserved regions flanking the AUG regions of the start codon in eukaryotic genes (7). All here used techniques are PCR – based and after the enzymatic reaction data are obtained the genetic diversity and similarity matrices are developed, that further were systematized in a phylogeny UPGMA dendrogram.

Materials and methods

Total genomic DNA was isolated and purified from 300 µl of total blood samples of 15 Canis lupus familiaris L. individuals provided by Veterinary Clinics of BUASVM form Timisoara, Romania. The breed of dog individuals was registered as the owner declared (Table 1).
Table 1

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<td>Amstaff</td>
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<td>14</td>
<td>N</td>
<td>Springer Spaniel</td>
</tr>
<tr>
<td>15</td>
<td>O</td>
<td>Wirehaired Teckel</td>
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</table>

The quantity and quality of DNA was assessed by specrophotometric measurement (NanoDrop 8000, Thermo Scientific). By serial dilution the DNA samples were brought to a concentration of 50 ng/ul. Three systems of molecular markers ISSR, DaMD and SCoT, were used for the genetic diversity assessment.

Table 2

<table>
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<th>No.crt.</th>
<th>Primer code</th>
<th>Sequence 5’…3’</th>
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<td>TGTGTGTGTGTGTGTC</td>
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<tr>
<td>8</td>
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<td>DAMD molecular markers</td>
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<tr>
<td>16</td>
<td>14C2</td>
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The primers used in this study were selected after an initial screening. The screening consisted of PCR amplification of a bulk DNA sample with 10 primers (Table 2) of each molecular markers system. The primers were randomly selected from the collection of Molecular Genetics Laboratory of BUASVM from Timisoara, Romania. The selected primers are highlighted in Table 2.

PCR was carried out in final reaction volumes of 25 µl containing 100 ng of DNA template. The composition of amplification mixture was carried out according the producer instructions for DreamTaq Green PCR Master Mix (2X) commercial kit (Thermo Scientific, Lithuania). The reaction was performed on a DNA Engine Peltier Thermal Cycler (MJ Research, U.S.A.) and the PCR program consisted of a first denaturing step for 5 min at 94°C, followed by 45 cycles of denaturation at 94°C for 45 - 60 sec, annealing at 48°C- 55°C for 45 - 60 sec and extension at 72°C for 2 min, the final step of extension at 72°C for 5 min., according to literature (3, 4).

The resulting PCR products were run on 1.6 % agarose gels in TAE buffer at a constant voltage of 100 V for 100 minutes.

The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England). The obtained data were analyzed with VisionWorksLC software (UVP, England).

The dendrogram was assessed from a set of variables by using DendroUPGMA (18), software. The Jaccard coefficient has been used to compare between sets of variables. The program calculates a similarity matrix and transforms similarity coefficients into distances and makes a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.
Results and discussion

Specific literature data does not contain information to state that here presented three markers systems were used in scoring genetic diversity of the dogs breeds. Only microsatellic molecular markers were previously used in similar studies (2, 10, 13). However, the VNTR markers systems have been successfully used in genetic diversity studies of other vertebrates (1, 3, 4). The lack of information lead to the first step of our study, the initial screening with 10 primers designed to target for each of the three markers systems. The goal of this step is to reveal those markers that will provide the most informative data in terms of good amplification pattern and polymorphism indication. A bulked DNA sample consisting of 5 µl of each dog individual DNA was used as template for PCR amplifications. For each primers set the PCR was separately carried out. Based on the screening obtained result, five primers for each molecular markers system were considered as a valuable choice for further studies (Figure 1), (Table 2). Primers that yielded faint or low number of amplicons were eliminated from the experiment. Therefore, the DNA fingerprinting study was developed on the basis of 15 markers system. After the primers selection, separate PCR amplifications were carried out targeting the selected molecular markers for each individual taken into study (Figure 2). The obtained raw data are summarized in Table 3.

Fig. 1. PCR based screening with primers designed for proposed molecular markers systems: Panel A: ISSR markers; Panel B: DAMD markers; Panel C: SCoT markers; M- Molecular weight marker – 100 bp DNA Ladder RTU, GeneDirex.
Table 3

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Total/percentage of polymorphic alleles 294/328 (86.63 %)

In this experiment, using data acquired from 15 polymorphic molecular markers, a binary matrix of 328 scored PCR products, from which 294 were polymorphic, was developed. SCoT primers yielded the highest number of amplicons, compared to ISSR and DAMD primers, and overall they exceeded also in terms of polymorphic band percentage (Table 3).

![DNA fingerprint](image-url)

Fig. 2. DNA fingerprint obtained using DAMD primer HBV 3: A - O dog individuals; M - Molecular weight marker - 100 bp DNA Ladder RTU, GeneDirex
Those differences are occurring because of their different sequence and DNA specific target. However, SCoT molecular markers are relatively new developed and have no history of being used intensely for this kind of experiments, but, as revealed by the obtained data those markers can be successfully used in establishing genetic diversity among small groups of animals. Nevertheless, when those three sets of data are combined the result can be more accurate and precise than in the case of using just one marker system. From the data resulted from the analyses of PCR results a binary matrix for each individual was developed. The binary matrix was used in assessing the similarity matrix and the genetic distance matrix using the Jaccard similarity coefficient (Table 4, 5).

The genetic distances values are used to reveal the genetic divergence between analyzed individuals. Thus, a value of 0 means no genetic divergence, and it is obtained only in the case when comparing an individual to itself, the value 1 being a theoretical maximum threshold that is impossible to be achieved in practice. The medium value is around 0.5. Analyzing the obtained matrix it can be observed that the individuals are, on average closely related, as expected, all the obtained values were around the medium value or beneath. The minimum value of 0.224 was obtained in this case is between individuals E and F, those being the two caucasian shepherds. The higher value of 0.581 is recorded between individuals A and L, those being viszla and poodle.

The genetic similarity is somehow reduced the highest value – 0.776, being registered between the individuals E and F, the Caucasian shepherds. As it can be seen in Table 5, the values for similarity hardly exceed the medium threshold, especially in the case of different groups of dogs. The lowest value of genetic similarity is 0.434, and it was recorded in the case of fox terrier (I) and springer spaniel (N).
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Table 4
Genetic distance matrix of the studied 15 dog individuals
### Table 5

Similarity matrix of the studied 15 dog individuals

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Fig. 3. UPGMA Dendrogram of *Canis familiaris* L. individuals created by DendroUPGMA program using data from the three molecular markers systems.

On the basis of resulted genetic similarity and distance matrices an UPGMA dendrogram was developed (Figure 3). The similarity coefficients are the values used for grouping the individuals in to clusters. The length of the clusters is being dictated by the genetic distance coefficients. Dendrogram analyses revealed that the 15 individuals are grouped in two different major clusters. The first cluster contains the individuals from O, N, L, M, I and K, namely Teckel, Springel Spaniel, Poodle, Fox Terrier and West Highland Terrier. According to the UK classification of dog breeds (the official Kennel Club of the United Kingdom)(17), into groups the springer spaniel is included into the Gundog group along with Viszla and poodle, but from our data the Viszla (A) individual is closely related with the German brack (J), and both are included in the second cluster beside the Pastoral, Utility and Hound dog groups. The Terrier group is represented in our study by the Fox terrier (I), West Highland Terrier (K) and according to the phylogeny the Amstasff (C).

However, from our study resulted that the C individual is more similar with the American bull dog (B), being clustered together and belonging to the big dog second cluster. The Saint Bernard (D) is found to be more related with the Pastoral
dog Group, namely the Caucasian Shepherds (E and F), but it belongs to the Working group. The Sarplaninac (G) is closely genetically related with the Kangal (H), both belonging to the Pastoral group. However, the first one is not widely recognized as a dog breed. In the case of Poodles dogs (L and M), the classification into groups is dependent of the breed variety. The standard variety is included in Hound dog group along with Teckel and Viszla, but the smaller variety are included in Gundog and Toys groups. According to obtained data the analyzed poodles are clustered along with the small dog’s clusters.

Conclusions

This paper presents an incipient study with the goal of developing a method based on molecular markers system and biochemical technique that can become an important tool of reveling the phylogeny of dog breeds. This method can became of great help in establishing the similarities among different breeds and the individuality of a pure breed. Moreover, the method can be applied also in the case of establishing the appurtenance of an individual to a pure breed, when standardized reference is available. The proposed molecular markers systems were proved to be reliable and provided accurate information in terms of establishing a molecular fingerprint but also in clustering individuals according to the degree of genetic similarity. Even so, most accurate data will be obtained by similar studies with standardized biological material of certified provenience. The developed similarity and genetic distance matrices differentiated among studied individuals according to their genetic inheritance, which was proved to be different from the existing grouping according to the breed usage.

Acknowledgements

This study was realised using the support and infrastructure project “Dezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinii veterinare şi tehnologiilor inovative pentru RO 05”, cod SMIS-CSNR 2669.

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EVALUATION OF IMMUNE RESPONSE IN SHEEP AFTER VACCINATION AGAINST CONTAGIOUS AGALACTIA

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Summary
In this study, aiming to evaluate the immune response in sheep after the vaccination against contagious agalactia, an ELISA method was developed for the detection of the antibodies against Mycoplasma agalactiae. The method was checked using negative and positive sera sampled from unvaccinated and vaccinated sheep against contagious agalactia, tested by a commercial ELISA kit used as reference. The optimal testing parameters as well as the interpretation criteria were set up, and the results were in agreement with those of the reference kit regarding the sensitivity and specificity. The specific vaccine induced antibodies were detected starting with the 7th day after the first vaccination, and the complete immune response was estimated at 21 days after the 2nd vaccination. The kinetics of post-vaccinal antibodies showed a rapid seroconversion in vaccinated animals, reaching 100% after 14 days post 2nd vaccination. This method can be used to monitor the immune response of sheep vaccinated against the contagious agalactia.

Key words: contagious agalactia, vaccine, antibody, ELISA

The contagious agalactia (CA) is an infectious disease caused by Mycoplasma agalactiae (MA) with implications in the pathology of small ruminants. CA can be considered as a syndrome caused by several species of mycoplasmas (12). Mycoplasma agalactiae is the main pathogen isolated in 90% of outbreaks in goats and 100% in sheep; other types as Mycoplasma mycoides subsp. mycoides Large Colony, Mycoplasma capricolum subsp. capricolum and Mycoplasma putrefaciens induce similar clinical signs in goats (1, 12).

The disease primarily affects the mammary gland, joints, eyes, and to a lesser extend the respiratory tracts, being characterized by mastitis, arthritis and keratoconjunctivitis (4).

The disease is widespread being detected mainly in the geographical areas in which the sheep milk is used for human consumption, causing economic losses due to the reduction or suppression of milk production; the infection develops as a herd enzootic infection, being able also to cause abortion and mortality in adult animals (7). In Romania, the incidence of the disease was reduced after the introduction of immunoprophylaxis, currently the disease evolving in unvaccinated herds (4).

The disease is notifiable, subjected to the quarantine measures and recorded in the OIE list of animal diseases.
The diseases caused by *Mycoplasma* can be eradicated by stamping out but this is not always practical, especially in the developing countries (10). The antibiotherapy may bring substantial improvements, however the etiologic agent is not completely eliminated from the body. Practice has shown that the implementation of immunoprophylaxis programs combined with general preventive measures offers the possibility to control the disease in the affected areas.

The specific prophylaxis of contagious agalactia is based on live attenuated or inactivated vaccines. Sheep vaccination against contagious agalactia induces humoral and cellular immune response, regardless the type of vaccine used (8).

The live attenuated vaccines elicit good immunity, but may produce transient infections associated with dissemination of the pathogen in milk when administered on lactating animals (4). The inactivated vaccines are safer and do not induce side effects after vaccination.

The most used vaccines are the inactivated ones, with aluminum hydroxide as adjuvant. The contagious agalactia immunoprophylaxis in Romania is based on the use of such a vaccine. The immune response in goats particularly encouraged attempts to use oil adjuvant inactivated vaccines, with satisfactory results (2,3).

The control of contagious agalactia implies, together with immunoprophylaxis means the existence of effective diagnostic tests with adequate specificity and sensitivity values. Thus, the evaluation of postinfectious or post vaccination seroconversion is necessary in establishing the incidence and prevalence of infection in susceptible livestock; demonstrating the post vaccine antibody level is important as a mean to assess the efficacy of vaccination against contagious agalactia. Worldwide, for the diagnostic or monitoring of the disease, several serological methods are available as the growth inhibition test, ELISA, complement fixation test, blotting techniques; during the last time, the molecular biology techniques (PCR rPCR) were developed and used more often. According to OIE, ELISA, complement fixation and immunoblotting techniques are recommended as standard serological tests which should be used to evaluate the humoral immune response (9). As a result there were described and compared several ELISA kits for the detection of antibodies specific to MA, using as antigen a total extract of *Mycoplasma agalactiae* or fusion proteins such as P48, P80 or P30 (6,12,13,14). A variant of ELISA based on monoclonal antibody was used to detect *Mycoplasma agalactiae* antigen in different samples (15).

The aim of this work was to evaluate the humoral immune response in sheep after vaccination with the inactivated vaccine Agavac, manufactured by Pasteur Institute, and in subsidiary to develop a specific ELISA method.
Materials and methods

Animals

The experiment was performed on a farm, over a period of six months. Thus, 75 Ille de France sheep of 50-75 kg and 4 – 24 months old were recruited for the study. The sheep were selected from herds free of contagious agalactia. Before the experimental study, the animals were clinically examined for pathologies involving the mammary gland and eyes, and tested negative for *M. agalactiae* serum antibodies by ELISA test. Antibiotherapy or any other specific treatment against mycoplasma were not administrated before or during the experiment. The trial was performed according to the Romanian and European animal health and welfare regulations.

Experimental design

The animals were vaccinated against contagious agalactia with Agavac, inactivated vaccine adjuvanted with aluminium hydroxide, containing *M. agalactiae* strain AG6, manufactured by Pasteur Institute. Three commercial batches of vaccine were used. The animals were randomly divided into three groups A, B and C of 25 sheep each, corresponding to each batch of vaccine. Within each group, 20 animals were vaccinated twice against CA (groups A-vacc, B-vacc and C-vacc) and the remaining five animals from each group were the unvaccinated control groups (A-Ctrl, B-Ctrl, C-Ctrl).

The vaccine was administered subcutaneously, one dose (1 ml) / animal, with a booster at 14 days after the first vaccination.

After vaccination, the animals were regularly monitored for the occurrence of the local adverse reactions, with particular regard to the body temperature and to the clinical status of mammary lymph nodes, joints, conjunctives and corneas.

Serology

For each group of animals blood samples were taken prior to the first vaccination (considered as samples at zero day). For group A, blood samples were collected as follows: 7 days after the first vaccination, and 7, 14 and 21 days after the 2nd vaccination (after booster). For groups B and C, blood samples were taken only at 21 days post 2nd vaccination (after booster).

The detection of antibodies specific to *M. agalactiae* in the sheep sera was performed by an indirect ELISA, using a kit developed in the Institute Pasteur (in-house) and *Mycoplasma agalactiae* Antibody Test Kit Idexx, used as a reference.

ELISA Idexx was used according to instructions for use provided by the manufacturer.

The ELISA procedure developed by Pasteur uses a whole bacterial antigen of *M. agalactiae* strain AG6, selected on the basis of antigenic characterization (11) in order to guarantee the presence of the main epitops, and which are found in the constitution of Agavac vaccine.
The antigen preparation was based on the protocol described by Pascale (11), with some modifications. *M. agalactiae* cultures of 72 hours were centrifuged at 10000 g, 4°C for 35 min. The bacterial pellet was washed twice with PBS (pH 7.2-7.4) by centrifugation at 13500 g, 4°C for 35 min. The final pellet was resuspended in PBS and constitutes the ELISA antigen. The protein concentration was estimated using Qubit® Protein Assay Kit (Invitrogen™/ Thermo Fisher Scientific Q33211).

The *M. agalactiae* antigen was used to coat the ELISA microplates (Nunc MaxiSorp), being diluted in bicarbonate buffer pH 9.6 to an optimal concentration in order to obtain the best discrimination between positive and negative samples (100µl/well, incubation at 37°C for 60 min). The coated plates were washed with PBS + 0.5% Tween 20 (PBST), treated with stabilizer and stored at 4°C.

The sheep serum samples were diluted 1/50 in PBST + 1% BSA buffer, added to the coated plates (100µl/well) and incubated for 30 min at 37°C. After incubation, the plates were washed three times with PBST buffer and 100µl of Monoclonal Anti - Goat/Sheep IgG – Peroxidase Conjugate (Sigma Aldrich) was added to each well at a dilution of 1:5000. The plates were incubated at 37°C for 30 min. After repeated washes, 100µl of substrate 2,2’-Azino-bis Diammonium (Calbiochem) diluted 1.5% in citrate-phosphate buffer (pH 5) with hydrogen peroxide were added to each well and incubated for 15 min, at room temperature in the dark. The reaction was stopped with 1.5% NaF solution and the optical densities (OD) were read at 405 nm (Labsystems Multiskan EX automatic reader).

The results were expressed both in optical density values, as well as a positivity ratio (S/P - Sample / Positive) or ELISA units (EU = S/P x 100). The negative cut-off was determined at S/P of 0.5 (50 EU), whereas the positive cut-off was established at S/P of 0.6 (60 EU). The results interpretation was carried out according to the criteria shown in Table 1.

<table>
<thead>
<tr>
<th>S/P</th>
<th>EU (S/P x 100)</th>
<th>Interpretation</th>
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<tbody>
<tr>
<td>≤ 0.5</td>
<td>≤ 50</td>
<td>Negative</td>
</tr>
<tr>
<td>0.5 &lt; S/P &lt; 0.6</td>
<td>50 &lt; EU &lt; 60</td>
<td>Suspect</td>
</tr>
<tr>
<td>≥ 0.6</td>
<td>≥ 60</td>
<td>Positive</td>
</tr>
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</table>

Results and discussions

During June to November 2016 there were carried out monitoring studies of the humoral immune response in sheep after vaccination with Agavac - inactivated vaccine against contagious agalactia of sheep and goats.

The experimental animals were clinically examined before and after vaccination. No clinical signs specific to contagious agalactia were noted, and no adverse local reactions at the site of injection were observed.
Sera from sheep vaccinated with the three batches of Agavac were tested with respect to detection of specific antibodies by immunoenzymatic technique, using the Idexx kit comparatively to the method / kit developed by the Pasteur Institute Bucharest.

The two ELISAs methods provided similar results for both the vaccinated and unvaccinated control groups (sera at 0 days).

The repeatability and reproducibility were calculated with replicates on the same plate or in different plates; thus, the obtained coefficient of variation were less than 15%, indicating that these parameters are appropriate.

The assessment of the specific humoral immune response for group A was achieved in kynetics, serum samples being collected prior to vaccination, at seven days after the first vaccination, and then at seven, 14 and 21 days after booster. Prior to vaccination, all tested animals were negative. At seven days after the first vaccination and seven days after the 2nd vaccination, 6/20 serum samples were negative and the antibodies against *M. agalactiae* were detected for 14/20 samples, by both ELISA methods / kits. Instead, at 14 and 21 days after booster, ELISA Idexx recorded 18 positive results (90%), two animals within the vaccinated group being further negative / suspect. ELISA Pasteur showed the presence of specific antibodies (positive results) in 20/20 vaccinated animals, with a positivity range of 67% - 118%, close to the ELISA Idexx. The average of the antibody titers expressed as ELISA units (EU) are shown in Figure 1.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Group A – The kinetics of post vaccinal *M. agalactiae* antibody. ELISA Idexx and ELISA Pasteur Institute (PI)

For groups of animals B and C serum samples were taken prior to the first vaccination and at 21 days after booster. Similar results were obtained and the average antibody titers after the vaccination are shown in Figures 2 and 3.
Fig. 2. Group B - average antibody titer expressed as ELISA units. ELISA Idexx and ELISA Pasteur Institute. T0 – day before vaccination; T21 pvII – 21 days after the 2nd vaccination.

Fig. 3. Group C - average antibody titer expressed as ELISA units. ELISA Idexx and ELISA Pasteur Institute. T0 – day before vaccination; T21 pvII – 21 days after the 2nd vaccination.

For the groups of sheep B and C, the results were negative before the vaccination, by both ELISA Idexx and Pasteur method. The antibody level increased significantly at 21 days after booster, which was confirmed as well by serum samples taken from animals of group A at the same time. ELISA Idexx showed 90% positive results for group B-vacc (2 samples were classified as negative, respectively suspect) and 95% for group C-vacc (one negative sample). ELISA Pasteur identified all samples as positive for both groups of animals. The post vaccine antibody titers expressed in ELISA units (EU) were in the range of 67-
125 EU for ELISA Idexx, respectively of 65-128 for Pasteur method (≥ 60, positive cut-off).

The results obtained by the two ELISA procedures indicate that they are close regarding of the values and the interpretation, the small differences being probably due to the particularities of the \( M. \textit{agalactiae} \) antigen used. The ELISA Idexx kit uses a recombinant subunit antigen, namely the P48 protein of \( M. \textit{agalactiae} \), while the Pasteur method uses a whole bacterial antigen.

Sera from sheep vaccinated with Agavac had specific antibodies detectable by both methods, starting from the 7th day after the first vaccination, the full immune response being assessed at 21 days after the 2nd vaccination; sera harvested from animals in the control groups showed the lack of seroconversion and low levels of antibodies (<40EU).

The similarity of the results demonstrates the homogeneity of the vaccine batches used in this study, as well as the uniformity of the vaccination procedure. Several authors have reported that inactivated vaccines against contagious agalactia adsorbed on aluminum hydroxide, do not induce an longlasting immune response (3, 5), probably due to differences in antigenicity between vaccine strains and the circulating ones, the assessment of the immunity duration requiring further studies. The experience with this vaccine has imposed the recommendation of biannual vaccination of sheep milk, whereas the vaccination applied during the second period of gestation is essential in transmitting the maternal immunity to the newborns.

**Conclusions**

The ELISA procedure can be used to assess the specific seroconversion in sheep following the vaccination with inactivated vaccine prepared from cultures of \( \textit{Mycoplasma agalactiae} \).

The post vaccinal antibodies were detected at seven days after the first vaccination with Agavac, highlighting an early humoral immune response.

The comparative use of a commercial ELISA kit and an in-house ELISA method allowed to obtain similar results that can be used to assess the protective effect of antibody as follows: titers higher than 60 EU be considered as positive titers.

The in-house ELISA was used to evaluate the immune response in sheep after vaccination with Agavac, obtaining similar results with the commercial kit used as a reference.

Testing negative sera from unvaccinated animals demonstrated that both methods had the same degree of specificity.

The inactivated vaccine Agavac elicit an active immunization, as demonstrated by the detection of specific antibodies. The kinetics of the post vaccine antibodies showed a rapid seroconversion on vaccinated animals, reaching 100% after 14 days post the 2nd vaccination.
References


STATISTICAL ANALYSIS REGARDING THE RELEVANCY OF RATIO BETWEEN DIFFERENT SOMATIC CELLS IN DAIRY COW’S NORMAL AND ABNORMAL MILK

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Summary
It is very well known the fact that the utilization of indirect and direct tests on milk is mandatory for mastitis surveillance in dairy farms. Our previous researches pointed out that for a better vision on the mastitis types and their evolution in a dairy farm is indicated to use at least one indirect (R-Mastitis test, Mast-O-Test) and one direct test (bacteriological exam, milk cytogram, somatic cells count), even some of them are expensive regarding the time and resources. The aim of this study was to establish the relevancy of ratio between different somatic cells in normal and abnormal milk from dairy farms. For this purpose the collected samples were examined using three tests (R-Mastitest or Mast-O-Test and bacteriological exam), to indicate and classify them into three groups, as normal milk from healthy cows, abnormal milk from cows with subclinical mastitis, and abnormal milk from cows with clinical mastitis. The milk smears were stained by Leishmann method for differential somatic cells count and the obtained data were statistically analyzed. The values of the ratios between different somatic milk cells can represent an exceptional diagnostic significance for mastitis installation into the mammary gland.

Key words: milk, somatic cells, dairy, mastitis

Cattle clinical mastitis still represents a very costly disease in dairy farms, expressed by decreasing of milk production and profit, drug costs, extra labor, even by death of affected animals. On the other hand, subclinical mastitis, until its detection, silently reduces the milk production and quality bonuses.

The ideal aim to dealing with cattle mastitis is to prevent them from happening. However, even under the best prevention and control programs, mastitis still occurs in dairy farms.

The detection of mastitis is generally based on revealing of some indicators for mammary gland inflammation. Clinical mastitis is very easy to detect and is based on recognition of physical characteristics of milk secretion and udder aspects. The detection of subclinical mastitis still represents a major issue. Early diagnose of mastitis is mandatory because the changes of mammary gland tissues take place much earlier then they become apparent. Actually, various methods based on physical and chemical changes of milk are used for diagnosis of subclinical mastitis, but all of them present in the same time, advantages and disadvantages.
Materials and methods

In the stables, for individual mastitis detection, especially subclinical forms, we agreed to apply on the collected milk samples two indirect tests, namely R-Mastitest and Mast-O-Test (electrical conductivity) because they are easy to use, economical and rapid, also helping as to select the samples for bacteriological exams.

For bacteriological exam the milk samples were processed at the Department of Infectious Diseases from the Faculty of Veterinary Medicine Timisoara.

To identify the etiologic agents of mastitis and further investigations were studied only the monocultures or those who had at least two types of colonies, one of them being dominant. Cultures that exhibited mixed colonies were ignored because it is almost impossible to establish which infectious agents had the dominant role and which were opportunistic; the final diagnosis was polymicrobial mastitis.

In order to establish normal and pathological milk cytograms, in the Cytohistology techniques Laboratory of the Cell Biology - Histology and Embryology Department from the Faculty of Veterinary Medicine Timisoara were examined 145 cow’s milk samples from three farms. Milk cytograms were performed immediately after the bacteriological exam and consisted in making smears, stained by Leishmann method and reading them on the Olympus CX41 microscope, following the similar steps as for leukograms.

Reading results of the milk smears were statistically interpreted using a software program called EZANOVA.

Results and discussions

Examined milk samples were classified into three categories as follows:
- 25 samples with normal milk, considering that the milk secretion was normal from the organoleptic point of view and negative at the applied indirect and direct tests (R-Mastitest, electrical conductivity, bacteriological exam);
- 60 milk samples collected from cows with subclinical mastitis, considering that the milk secretion was apparently normal from the organoleptic point of view, but positive at least at one of the applied tests;
- 60 milk samples collected from cows with clinical mastitis, considering that the milk secretion was changed, with or without sediment and positively, at least at one of the tests mentioned above.

All obtained data regarding the distribution of somatic cells types from normal milk samples were summarized in table 1 and represented in the chart 1.
Table 1

<table>
<thead>
<tr>
<th>Specification</th>
<th>Mean (%)</th>
<th>Mean standard error (%)</th>
<th>Standard deviation</th>
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<tr>
<td>Neutrophils</td>
<td>30.0</td>
<td>±0.86</td>
<td>3.83</td>
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<tr>
<td>Eosinophils</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Basophils</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>22.4</td>
<td>±1.04</td>
<td>4.67</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>43.4</td>
<td>±1.10</td>
<td>4.94</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>4.1</td>
<td>±0.46</td>
<td>2.07</td>
</tr>
</tbody>
</table>

Macrophages / Polymorphonuclear neutrophils ratio = 0.74
Polymorphonuclear neutrophils / Macrophages ratio = 1.34
Lymphocytes / Polymorphonuclear neutrophils ratio = 1.44
Polymorphonuclear neutrophils / Lymphocytes ratio = 0.69
Macrophages / Lymphocytes ratio = 0.51
Lymphocytes / Macrophages ratio = 1.93

Fig. 1. Graphic representation of the milk cytogram for normal milk

Regarding the distribution of somatic cells in normal healthy milk, it was found that the lymphocytes population was very well represented with an average of 43.4% (± 1.10). In comparison with data from the scientific literature, our results regarding the distribution of lymphocytes in normal milk were found in accordance with those found by some researchers (4), but equally, do dissonant note with those obtained by others, in which the lymphocytes population was represented in average proportion from 2% to 28% (1, 5, 8, 9). In normal milk, polymorphonuclear neutrophils were represented in mean proportion of 30.0% (± 0.86). These results are in agreement with those obtained by some researchers (5, 9), but, as in the case of lymphocytes are inconsistent with those obtained by others, where the polymorphonuclear neutrophils are represented in mean proportion from 3% (2, 3) to 45% (8). Macrophages from normal milk were found in the mean average of 22.4% (± 1.04). In this case, our results were in disagreement with the scientific literature, most researchers considering that macrophages are shown in normal
milk in more increased proportion, from 30% to 88% (2, 3, 9). In milk smears, after the identification of at least 400 somatic cells in order to achieve milk cytogram, polymorphonuclear eosinophils and basophils were found sporadically, so we consider to be absent. Epithelial cells were found in low number, namely the average rate being 4.01% (± 0.46). In this case, our results are consistent with those described in the scientific literature; all other authors found the epithelial cells from normal milk in proportion with an average between 0 and 7% (3, 8, 9).

The scientific literature states that the ratio macrophages/polymorphonuclear neutrophils may indicate the health of the mammary gland (7). Thus, in normal milk, this ratio is between the limits of 0.5 and 1.4 (mean = 0.95). From our study, it appears that this report shows average value 0.74, with limits ranging from 0.5 to 0.98, according to data from the literature. Also, we made other relations between somatic cell types from normal milk, obtaining the following results, shown in the chart 2:

- Polymorphonuclear neutrophils/Macrophages ratio = 1.34 (with limits between 1.64 – 2.19);
- Lymphocytes/Polymorphonuclear neutrophils ratio = 1.44 (with limits between 1.34– 1.54);
- Polymorphonuclear neutrophils/Lymphocytes ratio = 0.69 (with limits between 0.47– 0.68);
- Macrophages/Lymphocytes ratio = 0.51 (with limits between 0.3 – 0.9);
- Lymphocytes / Macrophages ratio = 1.93 (with limits between 2.39 – 4.62).

These average values and their limits will be considered in comparison with those obtained for the other two categories (subclinical and clinical mastitis) in order to determine if there are other correlations that provide clues on the health of mammary gland.

Fig. 2. The mean values of the ratio between the leukocytes types in normal milk
(Mf = macrophages; Ne = neutrophils; Lm = lymphocytes)
All data obtained regarding the distribution of somatic cells types in milk from cows with subclinical mastitis were summarized in table 2 and shown in chart 3.

**Table 2**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Mean (%)</th>
<th>Mean standard error (%)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>62.1</td>
<td>±1.77</td>
<td>13.53</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.2</td>
<td>±0.26</td>
<td>0.51</td>
</tr>
<tr>
<td>Basophils</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>16.0</td>
<td>±0.75</td>
<td>5.70</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>19.6</td>
<td>±1.58</td>
<td>11.92</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>2.0</td>
<td>±0.23</td>
<td>1.75</td>
</tr>
</tbody>
</table>

**Macrophages / Polymorphonuclear neutrophils ratio = 0.25**
**Polymorphonuclear neutrophils / Macrophages ratio = 3.86**
**Lymphocytes / Polymorphonuclear neutrophils ratio = 0.31**
**Polymorphonuclear neutrophils / Lymphocytes ratio = 3.18**
**Macrophages / Lymphocytes ratio = 0.82**
**Lymphocytes / Macrophages ratio = 1.21**

Fig. 3. Comparison regarding the distribution of the milk cells in normal milk (1) and subclinical mastitic milk (2)

The milk samples provided from cows with subclinical mastitis presented an increased percentage of polymorphonuclear neutrophils in comparison to their weight in normal milk, from 30.0% to 62.1% (± 1.77). From the statistical point of view, this increase is highly significant (p <0.001). The population of lymphocytes in the case of subclinical mastitis was highly significant decreased in number (p <0.001) compared to their distribution in normal milk, from 43.4% to an average rate of 19.6% (± 1.58). The macrophages were found in the mean proportion of 16.0% (± 0.75), and in this case, the number is very significant decreased (p <0.001) compared to normal milk, where they have been represented the mean
proportion of 22.4%. In direct smears, after the identification of at least 400 somatic 
cells, it was found the appearance of polymorphonuclear eosinophils, which were 
found in the average rate of only 0.2% (± 0.26), at the same time we considered 
the basophils absent, because their low number. Also, the number of epithelial 
cells in the milk from cows with subclinical mastitis was very significant decreased 
(p <0.001), in comparison with normal milk, the mean percentage being 
represented by 2% (± 0.23).

In the case of milk from cows with subclinical mastitis the 
macrophages/polymorphonuclear neutrophils ratio was an average of 0.25, with 
the limits between 0.18 and 0.32. Decreasing average ratio is explained by the 
significant increase of neutrophils proportion in milk. This event occurred because 
in the mammary gland, the first specific defense process against bacterial agents 
was phagocytosis, which was carried out by polymorphonuclear neutrophils. The 
scientific literature only states that the values of the macrophages 
/polymorphonuclear neutrophils ratio less than 0.5 means an infectious process 
installation in the mammary gland.

In the case of subclinical mastitis, other values of ratios made between the 
classes of leukocytes were set out below and represented in the chart 4, compared 
with those for normal milk: 
- Polymorphonuclear neutrophils/Macrophages ratio = 3.86 (with limits 
between 2.72 to 10.52);
- Lymphocytes/Polymorphonuclear neutrophils ratio = 0.31 (with limits 
between 0.19 to 0.53);
- Polymorphonuclear neutrophils/Lymphocytes = 3.18 (with limits between 
2.79 to 8.87);
- Macrophages/Lymphocytes = 0.82 (with limits between 0.4 to 1.6);
- Lymphocytes/Macrophages = 1.21 (with limits between 0.97 to 1.18).

![Fig. 4. Comparison regarding the mean values of the relations between 
the leukocytes types in normal milk and subclinical mastitic milk (Mf = macrophages; 
Ne = neutrophils; Lm = lymphocytes)](image-url)
From these researches was to be noted the increase of the average values of polymorphonuclear neutrophils/macrophages ratio, and polymorphonuclear neutrophils/lymphocytes ratio, as compared to normal milk, about 3 times (from 1.34 to 3.86) and respectively about 4.6 times (0.69 to 3.18), which we consider that is an exceptional diagnostic significance and full statistical assurance for subclinical mastitis installation into the mammary gland.

The macrophages/lymphocytes presented a slightly higher mean value in comparison with normal milk value, from 0.51 to 0.82, emphasized that in subclinical mastitis occurred numerical decrease of lymphocytes population in relation with macrophages. Equally was reported an insignificant decrease of average value for lymphocytes/macrophages ratio, from 1.93 to 1.21. In the case of lymphocyte/ polymorphonuclear neutrophils ratio, as compared to normal milk, the significant decrease of the mean value was observed, thereof of about 4.6 times (from 1.44 to 0.31), which is explained by the increased number of neutrophils in milk from cows with subclinical mastitis at the expense of lymphocytes population.

All statistical data regarding the distribution of somatic cells types in milk samples from cows with clinical mastitis and comparisons to the other two cases were summarized in table 3 and chart 5. The statistical comparisons revealed the dynamics of somatic cells in normal and abnormal milk.

Table 3

<table>
<thead>
<tr>
<th>Specification</th>
<th>Mean (%)</th>
<th>Mean standard error (%)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>67.3</td>
<td>±2.12</td>
<td>16.00</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.8</td>
<td>±0.12</td>
<td>0.92</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.0</td>
<td>±0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Macrophages</td>
<td>12.5</td>
<td>±0.88</td>
<td>6.67</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>16.0</td>
<td>±1.75</td>
<td>13.18</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>1.3</td>
<td>±0.19</td>
<td>1.43</td>
</tr>
</tbody>
</table>

Macrophages / Polymorphonuclear neutrophils ratio = 0.18
Polymorphonuclear neutrophils / Macrophages ratio = 5.38
Lymphocytes / Polymorphonuclear neutrophils ratio = 0.26
Polymorphonuclear neutrophils / Lymphocytes ratio = 3.72
Macrophages / Lymphocytes ratio = 0.69
Lymphocytes / Macrophages ratio = 1.44
In the case of milk samples from cows with clinical mastitis, from the distribution of somatic cells point of view was found also the increase of polymorphonuclear neutrophils proportion. This increase was very significant ($p < 0.001$) compared to their distribution in normal milk, but insignificant ($p > 0.05$) than in cows' milk with subclinical mastitis, polymorphonuclear neutrophils average being represented by 67.3% ($\pm$ 2.12). The population of lymphocytes in clinical mastitis milk, lowering insignificantly their number ($p > 0.05$) in comparison with milk from cows with mastitis subclinical, but highly significant ($p <0.001$) compared to normal milk. In this case, the lymphocytes were represented in mean proportion of 18.0% ($\pm$ 1.75). In clinical mastitis milk, macrophages presented the mean proportion of 12.5% ($\pm$ 0.88). In this case, the decrease of the number of macrophages is highly significant ($p <0.001$) compared both to their distribution in normal milk and the one from cows with subclinical mastitis. The polymorphonuclear eosinophils were highly significant increased in number ($p <0.001$) in comparison to the other milk samples, being found in mean ratio of 0.8% ($\pm$ 0.12). Also, in direct smears made from milk from cows with clinical mastitis, the basophils occurred, which were represented in mean ratio of 0% ($\pm$ 0.01). The number of epithelial cells in milk from cows with clinical mastitis distinct significantly decreased ($p <0.01$) compared to that from subclinical mastitis milk and highly significant ($p <0.001$) compared to that from normal milk. They were represented in mean ratio of 1.3% ($\pm$ 0.19).

Our results regarding the distribution of somatic cells in milk secretion derived from cows with clinical mastitis were similar to those obtained by other authors. In most cases, the sources only mention briefly that in clinical mastitis, polymorphonuclear neutrophils percentage increased greater than 90% (6). Macrophages/polymorphonuclear neutrophils ratio presented the mean value of 0.18, with limits between 0.06 and 0.30. For clinical mastitis, the values of
other ratios made between classes of leukocytes were listed below and shown in chart 6, in comparison with those from normal and subclinical mastitis milk:

- Polymorphonuclear neutrophils/Macrophages = 5.38 (with limits between 6.5 to 34.97);
- Lymphocytes/Polymorphonuclear neutrophils = 0.26 (with limits between 0.06 to 0.46);
- Polymorphonuclear neutrophils/Lymphocytes = 3.72 (with limits between 7.67 to 28.56);
- Macrophages/Lymphocytes = 0.69 (with limits between 0.03 to 1.35);
- Lymphocytes/Macrophages = 1.44 (with limits between 0.84 to 1.21).

In the case of clinical mastitis was noted an increase of the average values of polymorphonuclear neutrophils/macrophages and polymorphonuclear neutrophil/lymphocyte ratio in comparison with normal milk by 4 times (1.34 to 5.38) and respectively by 5.4 times (from 0.69 to 3.72), which, as in the case of subclinical mastitis, can represent an exceptional diagnostic relevancy for the installation of an infection disease in mammary gland. In the case of lymphocytes/polymorphonuclear neutrophils ratio, in compared to normal milk, it was shown very pronounced decrease of the average value hereof, is about 5.4 times (to 1.44 to 0.26). Macrophages/lymphocytes ratio presented a slightly higher mean value compared with normal milk, from 0.51 to 0.69 underlined by the fact that in subclinical mastitis there is a decrease in number and percentage of lymphocytes population. Equally significant decrease was found for average value of lymphocytes/macrophages ratio, from 1.93 to 1.44.

Fig. 6. Comparison regarding the mean values of the relations between the leukocytes types in normal milk and subclinical and clinical mastitic milk (Mf = macrophages; Ne = neutrophils; Lm = lymphocytes)
Conclusions

Polymorphonuclear neutrophils and lymphocytes populations presented the largest variation in milk cytograms carried out from cow’s milk both with subclinical and clinical mastitis.

It is true that the absolute number of leukocytes in the secretion of milk increases significantly in the case of infections of the mammary, to very high, exceeding one million/ml and, at the same time, increases the value of the relationship between neutrophils and the rest of the classes of leukocytes.

The cytological examination can serve in the diagnosis of cattle mastitis, providing not only a still image - as opposed to simply counting of milk somatic cells - but also a dynamic image, even some indications on the state of infectious disease and its evolution trend.

Acknowledgements

This study was realised using the support and infrastructure project “Dezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinii veterinare şi tehnologiilor inovative pentru RO 05”, cod SMIS-CSNR 2669.

References

PROTEIN CONTENT DEVIATIONS OF SUNFLOWER MEAL, RAPESEED MEAL AND SOY MEAL COMPARED WITH SPECIALIZED REFERENCES OF NUTRITIONAL VALUES AVERAGE

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Summary
Nutritional values of feed from specialized references are just some average values. They are determined based on several samples taken from a particular geographic area, within a certain timeframe. Samples taken from farms in the western part of Romania were analyzed and compared with values from the literature. It has been found that deviations occur in less up to 6.87% and 5.54% in addition to sunflower meal. Deviations from soybean meal were 8.59% in minus and 6.01% extra. Deviations values were highest in rapeseed meal reaching 29.8% (p<0.050). Because each area has specific soil conditions, climate, tradition or preference for certain varieties, pathology specific area, economic resources and scientific knowledge with different levels result that these results are valid only in certain areas and with short life. If you add and environmental factors (especially global warming), influencing the chemical composition of raw feed, results that these values have strict orientation value and should be determined for each sample in hand, especially when calculating feed rations given to a large number of animals.

Key words: nutritional values, protein, meals.

Many books have been written about the nutritional value of feed, sometimes in several volumes. Each prestigious nutrition institute in the world (NRC, INRA, OKIT, etc.) has its own reference values (2,8,9).

Determining the nutritional value of feed is a very complex process that involves several stages. These include: determination of gross chemical composition, digestibility, energy value and biological value of protein.

Since there are a number of peculiarities of environmental conditions, how harvest, preserve is and prepares feed, factors dependent on the food-bearing and of animal, all of this introduces many variables into the system. It follows that all these factors mark the nutritional value of feed.

Because each area has specific soil conditions, climate, tradition or preference for certain varieties, pathology specific area, economic resources and scientific knowledge with different levels, result that these results are valid only in
certain areas and with short life. If you add and environmental factors (especially global warming), influencing the chemical composition of raw feed, results that these nutritional characteristics can be very different. In order to keep in mind that in the growth of industrial animals every percentage counts these analyzes must often be repeated, at least at the change of the burden, the supplier, etc.

**Materials and methods**

Feed samples consisting of oilseed meals (sunflower meal, rapeseed meal and soy meal) were taken from different farms located in the western part of Romania using the standard sampling method. These samples were analyzed and compared with values from the literature (4,6,7).

Some elements of gross chemical composition of the feed were determined using established methods: crude protein (CP % ) ( SR EN ISO 5983-1:2006); dry matter ( STAS 12186/87 ) and humidity ( 64 ISO 96: 2001) ( DM % and U%).

The results obtained were interpreted with the help of statistical methods and were processed using the Z test.

**Results and discussions**

The raw protein content is considered, in many situations, as one of the most important quality criteria in the appreciation of a feed, especially in the case of monogastric animals. In the case of checking compliance with the feed formulations after which the compound feed is prepared, the percentage of protein is usually the first tested. That is why we have harvested three groups of oilseeds meals from different farms in the western part of Romania.

The variations in crude protein values in the analyzed meals (sunflower meal, rapeseed meal and soy meal) are shown in Figure 1.

The results were compared with values from the literature (1,3,5). It has been found that deviations occur in less up to 6.87% and 5.54% in addition to sunflower meal. Deviations from soybean meal were 8.59% in minus and 6.01 % extra. Deviations values were highest in rapeseed meal reaching 29.8%.
The obtained values were compared to each other and then to the average of the values in the literature. The statistical analysis of the results is given in Table 1 for these three meals.

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Sunflower meal</th>
<th>Soybean meal</th>
<th>Rapeseed meal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>Mean = 35.067</td>
<td>Mean = 44.45433</td>
<td>Mean = 28.87</td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td>Standard Error = 0.272244</td>
<td>Standard Error = 0.372402</td>
<td>Standard Error = 0.3893</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>Standard deviation = 1.491144</td>
<td>Standard deviation = 2.039731</td>
<td>Standard deviation = 2.13</td>
</tr>
<tr>
<td><strong>Minimum</strong></td>
<td>Minimum = 32.63</td>
<td>Minimum = 40.86</td>
<td>Minimum = 25.27</td>
</tr>
<tr>
<td><strong>Maximum</strong></td>
<td>Maximum = 37.47</td>
<td>Maximum = 47.39</td>
<td>Maximum = 32.75</td>
</tr>
<tr>
<td><strong>Count</strong></td>
<td>Count = 30</td>
<td>Count = 30</td>
<td>Count = 30</td>
</tr>
<tr>
<td><strong>Confidence Level (95.0%)</strong></td>
<td>Confidence Level = 0.556802</td>
<td>Confidence Level = 0.761648</td>
<td>Confidence Level = 0.79622</td>
</tr>
<tr>
<td><strong>Coefficient of variation CV</strong></td>
<td>Coefficient of variation = 4%</td>
<td>Coefficient of variation = 5%</td>
<td>Coefficient of variation = 5%</td>
</tr>
<tr>
<td><strong>Confidence interval</strong></td>
<td>[34.51; 35.62]</td>
<td>[43.69; 45.21]</td>
<td>[28.07; 29.66]</td>
</tr>
<tr>
<td><strong>Average value in specialized literature</strong></td>
<td>Average value in specialized literature = 35.5%</td>
<td>Average value in specialized literature = 44.7%</td>
<td>Average value in specialized literature = 36.0%</td>
</tr>
<tr>
<td><strong>Z score</strong></td>
<td>Z score = 0.613</td>
<td>Z score = 0.366</td>
<td>Z score = 3.494</td>
</tr>
<tr>
<td><strong>Z test p&gt;0.05</strong></td>
<td>Z test p&gt;0.05</td>
<td>Z test p&gt;0.05</td>
<td>Z test p&gt;0.05</td>
</tr>
<tr>
<td><strong>p calculated</strong></td>
<td>p calculated = 0.544</td>
<td>p calculated = 0.707</td>
<td>p calculated = 0.0015</td>
</tr>
</tbody>
</table>
Between the determined values and the average value in the specialized literature there are no significant differences in the case of sunflower meal and soy meal because Z test values were higher than 0.05 (p=0.544 and respectively p=0.707).

For rapeseed meal registered significant differences (p=0.0015). High deviations from the average are thus demonstrated. Deviations from the average are shown by the value obtained using the Z test that is lower than 0.05.

These values, which show a greater deviation from the averages indicated in the literature, draw attention to the fact that if raw materials are used by relying only on the theoretically expected protein content, they can lead to inadequate combined feedingstuffs.

Conclusions

Nutritional values of feed from specialized references are just some average values.

Samples taken from farms in the western part of Romania, analyzed and compared with values from the specialized literature, showed that deviations to sunflower meal and to soybean meal were insignificant (p<0.050). Deviations values were highest in rapeseed meal reaching significant results (p<0.050).

Because a lot of factors influencing the nutritive value results that these numbers have strict orientation value and should be determined for each sample in hand, especially when calculating feed rations given to a large number of animals.

Acknowledgements

This study was realised using the support and infrastructure project "Dezvoltarea infrastructurii de cercetare, educație și servicii în domeniile medicininii veterinare și tehnologiilor inovative pentru RO 05", cod SMIS-CSNR 2669.

References


ISOLATION OF RABIES VIRUS IN MOUSE NEUROBLASTOMA CELL LINE (N2A)

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Summary

Nowadays, isolation of the rabies virus by the Mouse Inoculation Test (MIT) has been widely replaced in many laboratories by the Rabies Tissue Culture Infection Test (RTCIT), being the second most used reference technique in rabies diagnosis. This latter offers many advantages: inexpensiveness, less time-consuming, easy to use and highly sensitive. Moreover, it avoids the use of live laboratory animals. Over the years, several studies on RTCIT using different cell lines have demonstrated that, N2a neuroblastoma cells are the most sensitive strain. The aim of this study was to perform the isolation of rabies virus in mouse neuroblastoma cell line as an alternative to the MIT. A panel of 27 brain samples from Moldova region, Romania, was used. These samples were isolated in the North-East of the country (Botosani, Suceava, Iasi, Piatra Neamt, Vaslui, Bacau, Galati and Vrancea) between 2012 and 2016 from different animal species, namely: 1 cat (Felix catus), 3 dogs (Canis canis), 10 cows (Bos taurus), 1 deer (Capreolus capreolus), 1 wolf (Canis lupus), and 10 foxes (Vulpes vulpes). Prior to RTCIT testing, all samples were analysed for the detection of rabies antigen using the Fluorescent Antibody Test (FAT), the reference rabies diagnosis technique recommended by the OIE and the WHO, as described in "OIE Terrestrial Manual", 2013. RTCIT was performed on LabTek Chamber Slides, at 8x10⁵ cell concentration per ml, using the BioRad (France) conjugate, with a 48 hour incubation period, in 5% CO₂ incubator at 37°C. Two positive controls (CVS and EBLV-1) and a negative one were included in each test. All of 27 brain samples were positive by FAT, and were confirmed by RTCIT, resulting in a 100% correlation between the two methods. No positive or false negative results were recorded. RTCIT should always be performed when FAT results are found negative or in case of doubtful FAT result and should replace the MIT whenever possible. Providing fast and reliable results, it is indispensable in case of human potential rabies exposure and it could be helpful for the medical staff in orienting the vaccination schedule.

Key words: rabies virus, cell line, RTCIT
According to OIE and WHO, three reference techniques are recommended to be performed for the confirmation of rabies diagnosis in animals, namely: Fluorescent Antibody Test (FAT), Mouse Inoculation Test (MIT) and Rabies Tissue Culture Infection Test (RTCIT) (5).

The MIT, known as the oldest method used for rabies diagnosis in animals, was first developed by Webster and Dawson in 1935 and it is still used in some countries (1). The value of this technique is represented by its ability to detect small quantities of rabies virus in weakly positive samples which can lead to false negative FAT results (7).

Starting with 1973, many WHO Experts on Rabies have been recommended the need of further studies on finding the most sensitive cell line to perform the isolation of rabies virus, as an alternative to the MIT.

To date, numerous studies and comparisons have been conducted between RTCIT, FAT and MIT. Before a technique to be accepted as a recognized diagnostic test, its sensitivity must be rigorously compared to the reference tests (9). Thereby, development of a tissue culture procedure with the purpose of replacing the MIT must be sensitive as well (7).

The first study reporting the use of tissue culture in rabies virus characterization was performed in 1913 by Noguchi and Levaditi. With the time, several cell lines of neural origin (e.g: N2a), as well as some non-neural cells (e.g: CER (chick embryo-related), BSR, BHK-21, HEK-293, McCoy cells, bovine, bat, shunk, dog and raccoon cells) and protocols were experimented until today (3, 9, 2).

The baby hamster kidney (BHK-21) cell line was the first attempt for the isolation of rabies virus, but the results were not encouraging, with variable success rates and inferior sensitivity compared to MIT (9, 2).

Being a neurotropic virus, it is expected that its attachment to be more towards the membranes of nervous tissue origin than to membranes of non-neural origin (6). Nevertheless, in a study published in 2010 by Madhusudana et al, it was proven that the HEK-293 cell line (human embryonic kidney) is as sensitive as the N2a cell line and can be an alternative for rabies diagnosis.

Nowadays, isolation of the rabies virus by MIT has been widely replaced in many laboratories by the Rabies Tissue Culture Infection Test (RTCIT), being the second most used reference technique in rabies diagnosis. This latter offers many advantages: inexpensiveness, less time-consuming, easy to use, highly sensitive and the capacity to detect small amounts of virus (7). Moreover, it avoids the use of live laboratory animals.

The N2a murine neuroblastoma cells, derived from different clones were introduced for the isolation of the rabies virus from 1978 onwards (4). Over the years, several studies on RTCIT using different cell lines have demonstrated that, N2a murine neuroblastoma cells is the most sensitive strain, being at the moment the reference cell line recognized by the WHO and OIE (2).

The aim of this study was to perform the isolation of rabies virus in mouse neuroblastoma cell line as an alternative method to the MIT.
Materials and methods

Virus isolates. A panel of 27 brain samples from Moldova region, Romania, was used. These samples were isolated in counties from the North-East of the country (Botosani, Suceava, Iasi, Piatra Neamt, Vaslui, Bacau, Galati and Vrancea) between 2012 and 2016 from different animal species, namely: 1 cat (*Felix catus*), 3 dogs (*Canis canis*), 10 cows (*Bos taurus*), 1 deer (*Capreolus capreolus*), 1 wolf (*Canis lupus*), and 10 foxes (*Vulpes vulpes*).

Prior to RTCIT testing, all samples were analyzed for the detection of rabies antigen using the Fluorescent Antibody Test (FAT), the reference rabies diagnosis technique recommended by the OIE and the WHO, as described in "OIE Terrestrial Manual", 2013.

Table 1 shows the geographical origins of the samples, the source of each sample, year of isolation and host species.

<table>
<thead>
<tr>
<th>No.</th>
<th>Country</th>
<th>City</th>
<th>Host species</th>
<th>Code sample</th>
<th>Year of isolation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Romania</td>
<td>Barlad</td>
<td>Cat</td>
<td>DR1017</td>
<td>2014</td>
<td>SVFSD*VS</td>
</tr>
<tr>
<td>2</td>
<td>Romania</td>
<td>Vrancea</td>
<td>Fox</td>
<td>DR1018</td>
<td>2015</td>
<td>SVFSD VN</td>
</tr>
<tr>
<td>3</td>
<td>Romania</td>
<td>Vrancea</td>
<td>Fox</td>
<td>DR1019</td>
<td>2014</td>
<td>SVFSD VN</td>
</tr>
<tr>
<td>4</td>
<td>Romania</td>
<td>Vrancea</td>
<td>Fox</td>
<td>DR1020</td>
<td>2013</td>
<td>SVFSD VN</td>
</tr>
<tr>
<td>5</td>
<td>Romania</td>
<td>Vrancea</td>
<td>Wolf</td>
<td>DR1021</td>
<td>2014</td>
<td>SVFSD VN</td>
</tr>
<tr>
<td>6</td>
<td>Romania</td>
<td>Vrancea</td>
<td>Cow</td>
<td>DR1022</td>
<td>2013</td>
<td>SVFSD VN</td>
</tr>
<tr>
<td>7</td>
<td>Romania</td>
<td>Vrancea</td>
<td>Cow</td>
<td>DR1023</td>
<td>2013</td>
<td>SVFSD VN</td>
</tr>
</tbody>
</table>
Method description. RTCIT was performed on LabTek Chamber Slides (Thermo Fisher Scientific, USA), at $8 \times 10^5$ cell concentration per ml, using the BioRad (France) conjugate, with a 48 hour incubation period, in 5% CO$_2$ incubator at 37°C. Two positive controls (the challenge virus standard CVS 27 strain of rabies virus and EBLV-1) and a negative one were included in each test.

Materials. Both biological and chemical materials were used for the technique. The biological materials were represented by the 27 brain samples to test and by the two positive controls, namely: mouse infected with CVS 27, mouse infected with EBLV-1 and a negative one, represented by an uninfected mouse. The chemical materials needed were: Murine Neuroblastoma cells N2a (ATCC CCL-131), DMEM medium (Gibco, UK), Fetal Calf Serum - FCS (Sigma Life Science, UK), trypsin, Acetone 80% (Thermo Chemical, UK), BioRad conjugate (France), sterile Phosphate Buffered Saline (Sigma Life Science, UK), sterile Distilled Water (Sigma Life Science, UK) and Glycerol mounting medium (LightDiagnostic, USA).

Medium and inoculum preparation. The first step consisted in preparation of the DMEM medium (enriched with 1% of antibiotics: Penicilene, Streptomicine and Amphotericin; 2,5 µg/ml of antimycoplasmic Plamocin 0,02% and 5% of FCS) and distribution in 11,5 ml Rohren tubes of 4 ml previous prepared medium for each
samples to test. A 1/5 cerebral tissue suspension was prepared from 1 gram of sample and 4 ml of DMEM and centrifuged for 15 minutes at 3000 rpm/min (Thermo Scientific Multifuge X1R Centrifuge). After centrifugation, all the tubes were stored at 4°C until use.

Cell suspension preparation. The cell suspension was prepared using the murine neuroblastoma cells (N2a) at 8x10^5 cell concentration per ml and DMEM medium previously prepared. When the cell monolayer was up to 80% confluent, the cells were trypsinized and counted in Neubauer chamber (Optik Labor, Germany).

Inoculation and incubation. In each Labtek, for cells sedimentation, 400 µl of cell suspension were added and incubated at room temperature for 5-10 minutes. Consecutively, 50 µl of each brain suspension prepared were distributed in the wells as follows: the two positive and one negative controls usually intended in the first LabTek and the samples to test in the others. All the LabTek were incubated for 48 hours in a humid chamber with 5% CO2 incubator (SANYO) at 37°C. After the incubation period, the medium was discard from each well using a pump (Vacuubrand, Germany) and the slides were dried at room temperature for 10 min.

Acetone fixation and slides staining. All the slides were introduced in ice-cold acetone 80% and fixed at -20°C for 30 min. After fixation, the slides were dried at room temperature.

For slides staining, an anti-rabies conjugate was used and prepared according to manufacturer recommendations. The lyophilized conjugate was reconstituted in 3 ml of sterile distilled water and centrifuged for 5 min at 1500 rpm/min for clarification and the supernatant resulted was used for staining. Each well of the Labtek was filled with 50 µl of conjugate and then incubated at 37°C for 30 min.

Washing and mounting medium. All the slides were washed in sterile PBS and distilled water and dried at room temperature. One drop of the mounting medium was added on each slide and at the end the coverslips.

Microscope reading. The slide’s reading was performed using a fluorescence microscope (Olympus BX41) with x20 and x40 magnification. In order to validate the technique, the control slides examination is mandatory.

Results and discussions

Consecutively, all 27 brain samples were positive by FAT and confirmed by RTCIT, resulting in a 100% correlation between the two methods. Two positive controls (CVS 27 and EBLV-1) and a negative one were included in each test. No positive or false negative results were recorded.

The usual method of examining cell cultures for the presence of rabies virus is by immunofluorescence staining. For the slides staining a french conjugate (BioRad) was used. It is important to note that a qualitative conjugate is required for a good reading and result.
The technique is considered valid, when the fluorescence is detected on the examination of the positive controls CVS 27 and EBLV-1 (Fig. 1, 2) and when no fluorescence is detected on the negative control (Fig. 3).

To confirm the rabies diagnosis by RTCIT, a single infected and positive cell is considered sufficient (Fig. 4).

The intensity of the positivity of a sample with respect to another is also reflected and depending by the virus concentration found in each sample, some of them being more infected by the rabies virus than others (Fig. 5).

However, MIT has been considered for a long time the only technique that may confirm or not the diagnosis of rabies, because of the long observation period required, the increasing cost and the need to replace the use of animals in the laboratory, alternative techniques have been found (8).

It is known that, the decision to provide or withhold the post exposure vaccination in humans after a bite coming from a rabies suspected animal is frequently based on the results of the rabies antigen detection assays, provided by the regional laboratory (7).

Considering the fact that rabies has a high medical significance in humans and the disease can be prevented by timely postexposure vaccination (1; 10), even if false negative FAT results are not common, they may occur (6), thereby a fast, specific and very sensitive technique must be performed in addition and used as a back-up procedure to the FAT.
Conclusions

RTCIT should always be performed when FAT results are found negative or in case of doubtful FAT result and should replace the MIT whenever possible.

The isolation of rabies virus in N2a murine neuroblastoma cell line is at least as efficient as Mouse Inoculation Test, even in the case of small quantities of rabies virus.

RTCIT is a fast technique and reduces the time required for diagnosis from 10-21 days with MIT to only 48-72 hours.

Providing fast and reliable results, it is indispensable in case of human potential rabies exposure and it could be helpful for the medical staff in orienting the vaccination schedule.

References

RELATION BETWEEN TYPE OF WORK AND BEHAVIOUR IN WORKING HORSES

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Summary
The behaviour of working horses is important for both the safety and the work performance of these animals and of the people using them. The aim of this research was to investigate the relationship between the type of work the horses are used for and their human-related behavior. The study included 145 working horses of which 20 were used for hard work, 46 for medium work and 79 for light work. The behavioral response (general attitude, aggressiveness, fear/avoidance, indifference, friendliness) of the horses was assessed in relation to an unknown and a known (owner) human, by specific methods. The data were analysed using the SPSS statistical software. In all three tests (approach, passing near and touching the horse), both towards the unknown assessor and the owner, the horses used for hard work were more aggressive, more fearful and more indifferent compared with the other horses. The friendly response was observed most frequently in the horses used for light work. Significant correlations were found between the different behavioural responses of the horses and type of work they were used for. The friendliness of the horses showed positive correlations with light work, fearfulness with hard work and indifference with medium work. The results of the study indicated that the type of work may have significant influence on the behaviour of the horses.

Key words: Horse behavior, working horses, type of work

The behaviour of working horses is important for both the safety and the work performance of these animals and of the people using them. According to Boivin et al. (2) the interrelations between animals and their caregivers are acting like a double mirror: both the animal’s perception of the human and the stockman’s perception of the animal determine the quality of the stockman-animal relationship. This happens in horses maybe even more than in other species, because of their high adaptability and ability to use cognition from simpler forms of learning (such as classical and operant conditioning) to stimulus generalization, discrimination learning, even categorization learning and some degree of concept formation, as described by Hanggi (4). Working with horses may involve accidents described in many statistics. A two year long study in the United States (8) reports an estimate of 11502 people for each studied year suffering traumatic brain injuries from horse related incidents. The same study raises awareness that prevention programmes should target horseback riders and horse caregivers to educate participants about horse behaviour and proper handling of horses (8). Lately horses’ behaviour,
mental abilities and needs and their relationships with humans come more and more in focus. A recent study (9) about horse-related risk highlights risk mitigation strategies directed at improving the predictability of horses (through making horses more predictable and making people more able to predict their behaviour) as a less addressed but valuable tool in improving safety around horses. In this context the studies of horse behaviour and the human-horse relationship gain additional importance, adding new elements to a knowledge that brings benefits for the welfare of the horse and people around.

The aim of this research was to investigate the relationship between the type of work the horses are used for and their human-related behavior.

**Materials and methods**

The study included 145 working horses of which 20 were used for hard work (hard working horses HWH), 46 for medium work (medium working horses MWH) and 79 for light work (light working horses LWH) from two rural regions of Transylvania. The selection of the horses was made according to the type of work they were used for, with the agreement of the owners to be assessed. The behavioral response (general attitude, aggressiveness, fearfulness/avoidance, indifference, friendliness) of the horses was assessed in relation to an unknown (assessor) and a known (owner) human using three behavioral tests: approach, walk beside and chin contact test. Each horse included in the study was assessed by the methods described by Popescu and Diugan (7).

The data were analyzed using the SPSS statistical software. The Kruskal-Wallis nonparametric one-way ANOVA test was used for the comparisons and the Spearman’s rank coefficient to study the relationship between the behavioural reactions of horses and type of work. The value of minimal significance was considered at $P < 0.05$.

**Results and discussions**

The results obtained by assessing the horses’ behaviors towards known and unknown humans depending on the type of work they were performing (hard work, medium work and light work) by three tests (approach, walk beside and chin contact test) are presented in Tables 1 - 3. The prevalence of the behaviours was compared between horse categories.
Table 1

Human-related responses of 145 assessed horses in the approach test

<table>
<thead>
<tr>
<th>Behavioural response</th>
<th>% of horses displaying the human-related behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assessor</td>
</tr>
<tr>
<td>HWH (n=20)</td>
<td>MWH (n=46)</td>
</tr>
<tr>
<td>Aggressiveness</td>
<td>5.00</td>
</tr>
<tr>
<td>Avoidance/fear</td>
<td>25.00</td>
</tr>
<tr>
<td>Indifference</td>
<td>65.00</td>
</tr>
<tr>
<td>Friendliness</td>
<td>5.00</td>
</tr>
</tbody>
</table>

HWH = hard working horses; MWH = medium working horses; LWH = light working horses

The Kruskal-Wallis test did not show significant differences (P > 0.05) of the behavioural response between the three working horse categories (HWH, MWH and LWH) in none of the performed tests.

Table 2

Human-related responses of 145 assessed horses in walk beside test

<table>
<thead>
<tr>
<th>Behavioural response</th>
<th>% of horses displaying the human-related behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assessor</td>
</tr>
<tr>
<td>HWH (n=20)</td>
<td>MWH (n=46)</td>
</tr>
<tr>
<td>Aggressiveness</td>
<td>10.00</td>
</tr>
<tr>
<td>Avoidance/fear</td>
<td>65.00</td>
</tr>
<tr>
<td>Indifference</td>
<td>10.00</td>
</tr>
<tr>
<td>Friendliness</td>
<td>15.00</td>
</tr>
</tbody>
</table>

HWH = hard working horses; MWH = medium working horses; LWH = light working horses

Behavioral tests are considered a major component in welfare assessment of working horses. These tests identify fearfulness or aggression toward humans and can help to demonstrate the nature of the human-animal interaction.

In all three tests both towards the unknown assessor and the owner, the horses used for hard work were more aggressive, more fearful and more indifferent compared with the other horses. The friendly response was observed most frequently in the horses used for light work. The current findings are consistent with those reported by other researchers (1, 3, 7).
Table 3

<table>
<thead>
<tr>
<th>Behavioural response</th>
<th>% of horses displaying the human-related behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assessor</td>
</tr>
<tr>
<td></td>
<td>HWH (n=20)</td>
</tr>
<tr>
<td>Aggressiveness</td>
<td>10.00</td>
</tr>
<tr>
<td>Avoidance/fear</td>
<td>50.00</td>
</tr>
<tr>
<td>Indifference</td>
<td>30.00</td>
</tr>
<tr>
<td>Friendliness</td>
<td>10.00</td>
</tr>
</tbody>
</table>

HWH = hard working horses; MWH = medium working horses; LWH = light working horses

Significant correlations were found between the different behavioural responses of the horses and the type of work they were used for. The friendliness of the horses showed positive correlations with light work (observer approach, $r_s=0.17$; owner’s touch, $r_s=0.22$, $P < 0.05$), fearfulness with hard work (observer approach, $r_s=0.18$; owner approach, $r_s=0.21$; owner’s touch, $r_s=0.30$, $P < 0.05$) and indifference with medium work (observer approach, $r_s=0.16$; observer’s walk besides, $r_s=0.18$; owner approach, $r_s=0.22$; owner’s touch, $r_s=0.19$, $P < 0.05$). Similar results were reported by Popescu and Diugan (7) and Popescu et al. (6). These interactions were to be expected, as the risks of brutal human handling and health problems increase for hard-working horses (5).

Conclusions

The results of the study indicated that the type of work may have significant influence on the behaviour of the horses. Besides the fact that usually the hard working environment comprises accident risks by itself for horses and humans, impropriate human-animal relationship indicated by the animals’ aggressiveness or fearfulness may involve additional risk situations with increased likelihood for accidents. In conclusion better handling practices and improved human-horse relationships may bring benefits both for a better welfare of horses and increased safety for human workers.

References

GOATS’ WELFARE ASSESSMENT IN A FARM FROM NORTH EASTERN ROMANIA

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Summary

Compared to other farm species, goats’ welfare is a topic insufficiently approached at both national and worldwide levels. Therefore, this paper aims to study some objective welfare parameters for this species and to improve the knowledge on this particular issue. The research was conducted in an intensive-type farm of Bacau County with a livestock of 750 animals (does, bucks and kids). The animals were housed in a stable with inner space divided into a storage area, a central passage way with collective pens aside, dairy goat milking parlour at the end of the house, auxiliary spaces and area for stockmen. The lighting system was mixed and the ventilation was active. There was used deep straw bedding of 10-20 cm thickness, replaced on monthly regular basis. In order to establish the group welfare, there were first assessed some engineering-based parameters: space allowance, microclimate parameters (by using 360-type thermohygrometer; LX 1108 light meter; SL 4102 sound meter; Testo 405 V1 thermo-anemometer; Oldham MX 2100 gas analyser) and bedding quality (humidity and elasticity) by knee test. The assessment was completed with some animal-based parameters: body condition score, limb injuries / lameness prevalence, abnormal behaviour prevalence, blood biochemical panel. The results showed that space allowance was in accordance with the welfare standards, housing conditions were appropriate, except for some minor lighting deficiencies. The animal-based parameters indicated no significant issues (acceptable herd management, good body conditions scores, low prevalence of limbs injuries and less than 2% expression of abnormal behaviours). However, there could be noticed the lack of outdoor access and some blood biochemical panel changes, without clinical significance, probably caused by high protein diets. Following the results, the welfare level in the assessed farm was found acceptable. Further improvements addressing the noticed deficiencies can result in an optimum welfare level.

Key words: biochemical panel, goats, microclimate parameters, welfare assessment

Nowadays it is largely accepted by the scientific community, general public and also by farmers, transporters or animal source food processors that animal husbandry, transportation and slaughtering cannot be conceived without ensuring a good welfare level – as an essential condition for both higher production and maintaining the position in the worldwide animal source food market, but also a moral duty of man towards animals.

The most accepted and useful definition of animal welfare is stated by Broom, D.M. (1, 2): welfare of an individual is its physiological and psychological state as regards its attempts to cope with its environment. In this perspective, animal welfare can largely vary from very poor to excellent and could be objectively...
assessed on scientific basis, by addressing three conceptual fields: animal physiology, animal ethology and ecology (the influence of the environment, of the animal housing conditions).

It can be noticed an increasing demand for goat milk and goat milk products, which motivates more and more goat farmers to approach the intensive rearing system. Nevertheless, compared to other farm species, goats’ welfare is a topic insufficiently studied at both national and worldwide levels. Therefore, recent studies have attempted to fill this gap e.g. the studies included in European project AWIN – Animal Welfare INdicators for goats (9). This paper aims to study some objective welfare parameters for this species and to assess the level of welfare in a farm from North Eastern Romania.

**Materials and methods**

The researches were conducted during January – May 2016 in an intensive-type farm from Bacau County with a livestock of 750 animals of Murcia-Granada, Saanen, French, Anglo-Nubian, Carpathian breeds and hybrids (does, bucks and kids). The animals were housed in a stable of 150 m length, 22 m width and 6 m height. The inner space was divided into a storage area, a central passage way with collective pens aside, dairy goat milking parlour at the end of the house, auxiliary spaces and area for stockmen (figure 1). The lighting system was mixed: windows with a total surface of 49.44 sqm and 93 fluorescent tubes of 36 W and eight light projectors of 150 W. The house ventilation was mechanical, with four fans (two for air inlet and two for outlet). There was used deep straw bedding of 10-20 cm thickness, changed on monthly regular basis and disposed outside of the house, on a manure platform.

The assessment of goats’ welfare at group level was done on the basis of engineering-based parameters and animal-based parameters.

Fig.1. Goat house: outside view (left) and inner space division (right)
The parameters within the first class consisted in: space allowance, microclimate parameters and bedding quality (humidity and elasticity). In order to establish the values of different microclimate parameters, there were used the following devices: 360-type thermohygrometer for air temperature and relative humidity, LX 1108 light meter for light intensity and uniformity; SL 4102 sound meter for noises intensity; Testo 405 V1 thermo-anemometer for air drafts’ velocity; Oldham MX 2100 gas analyser for air ammonia and hydrogen sulfide concentrations. The quality of the bedding was established by applying a subjective exam with 2 stages: wet-knee test and drop-knee test.

The studied animal-based parameters were: the body condition score - assessed in 50 animals and scored from 1 to 5 points (8), the limb injuries / lameness prevalence, the abnormal behaviour prevalence and the blood biochemical parameters in three collected samples: the values of blood urea nitrogen (BUN), serum creatinine (CREA), total proteins (TP), albumin (ALB), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glucose (GLU), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), triglycerides (TRIG), creatine kinase (CK).

Results and discussions

In the goats’ house, the space allowance was in accordance with the welfare standards, the surface for each mature animals being of 2.5 – 6 sqm and for each young or kid of 0.5 – 5 sqm. It could be noticed a good promotion of social interactions among the goat groups by housing in the same pen animals from the same breed, with similar body weight or in the same lactation stage. However, as a deficiency, it could be noticed the lack of outdoor access, with great importance in goat welfare.

Regarding in-house air temperature, the values in the cold season were between 8°C and 18°C and in spring months between 13°C and 24°C. Regarding air relative humidity, the values were: 72.2% in January, 68.8% in February, 65.9% in March, 61% in April, 60.5% in May. These values framed within the admitted values: 5°C – 25°C and maximum 70% respectively (5, 6, 7).

The results obtained for light intensity (table 1) reveal some minor lighting deficiencies: in one pen the value being lower than the minimum admitted value: 20 lx and subsequently the uniformity coefficient being bellow 0.3 (the ratio of minimum value and maximum value of light intensity = 12/53 = 0.23). The sound intensity framed within the accepted values for houses with mechanical ventilation, all the obtained results being lower than 90 dB (table 2).

For air drafts’ intensity, all the obtained values were lower than the maximum admitted values (table 3), as well as for air ammonia and hydrogen sulfide concentrations. Thus, the ammonia concentrations ranged from 7 to 18 ppm - while the maximum admitted value is 26 ppm, the hydrogen sulfide concentrations ranged from 0 to 3 ppm - the maximum admitted value being 10 ppm.
### Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Measurement point</th>
<th>Obtained value ((lx = \text{lux}))</th>
<th>Recommended values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pen for does close to kidding period</td>
<td>53 lx</td>
<td>• minimum 20 lx (national recommended values)</td>
</tr>
<tr>
<td>2</td>
<td>Pen for dairy goats</td>
<td>46 lx</td>
<td>• minimum 100 lx (other recommended values in the literature - 5, 6)</td>
</tr>
<tr>
<td>3</td>
<td>Maternity pen for does</td>
<td>27 lx</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pen for young goats</td>
<td>28 lx</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pen for nannies with kids</td>
<td>23 lx</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Pen 1 for pregnant goats</td>
<td>12 lx (\downarrow)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Pen 2 for pregnant goats</td>
<td>24 lx</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>No.</th>
<th>Measurement point</th>
<th>Obtained value ((dB = \text{decibels}))</th>
<th>Recommended values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pen for bucks</td>
<td>60 dB</td>
<td>• maximum 50-60 dB (natural – wind driven – ventilation system)</td>
</tr>
<tr>
<td>2</td>
<td>Maternity pen for does</td>
<td>58 dB</td>
<td>• maximum 80-90 dB (mechanical ventilation)</td>
</tr>
<tr>
<td>3</td>
<td>Center of the house</td>
<td>56 dB</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pen for dairy goats</td>
<td>57 dB</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pen for young goats</td>
<td>59 dB</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Milking hall</td>
<td>72 dB</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>No.</th>
<th>Measurement point</th>
<th>Obtained value ((m/s))</th>
<th>Recommended values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maternity pen for does</td>
<td>0.1 m/s</td>
<td>• maximum 0.1-0.5 m/s (mature goats)</td>
</tr>
<tr>
<td>2</td>
<td>Pen 1 for pregnant goats</td>
<td>0.1 m/s</td>
<td>• maximum 0.2 m/s (young goats)</td>
</tr>
<tr>
<td>3</td>
<td>Pen for does close to kidding period</td>
<td>0.2 m/s</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pen for young goats</td>
<td>0.1 m/s</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pen 2 for pregnant goats</td>
<td>0.2 m/s</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Pen 3 for pregnant goats</td>
<td>0.1 m/s</td>
<td></td>
</tr>
</tbody>
</table>

Concerning the quality of straw bedding, the result of the wet - knee test is negative: the examiner knees haven't moistened within a 10 seconds period contact with the floor. The result of drop - knee test is also negative: the examiner didn't feel any pain when crouching and then dropping to the knees on the goat pens. It can be concluded that the bedding is dry and soft enough to ensure comfort for goats (4).

Within the second class of studied welfare parameters (namely the animal-based parameters), the values for body condition score were presented in figure 2. The body condition has an average values of 2.4 for 50 animals, which indicates a good feeding management.
The limb injuries / lameness prevalence was considered insignificant, being observed some minor lameness only in three individuals.

There were not noticed major abnormal behavioral patterns, except from some sporadic wood chewing in some animals. It is worth to mention here that in several goat pens was applied environment enrichment, by giving the animals the possibility to climb on different surfaces.

The results of blood biochemical analysis are shown in table 4.

Table 4

<table>
<thead>
<tr>
<th>Parameter – M.U.</th>
<th>Reference value (3)</th>
<th>1&quot; sample</th>
<th>2&quot; sample</th>
<th>3&quot; sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN - mg/dl</td>
<td>10-21</td>
<td>22.8↑</td>
<td>26.9↑</td>
<td>29.12↑</td>
</tr>
<tr>
<td>CREA - mg/dl</td>
<td>0.6 - 1.4</td>
<td>0.58</td>
<td>1.61↑</td>
<td>0.72</td>
</tr>
<tr>
<td>TP - g/dl</td>
<td>6.4-7.8</td>
<td>7.51</td>
<td>8.56↑</td>
<td>9.02↑</td>
</tr>
<tr>
<td>ALB - g/dl</td>
<td>2.8-3.8</td>
<td>3.31</td>
<td>2.9</td>
<td>3.84</td>
</tr>
<tr>
<td>ALT-U/l</td>
<td>23-44</td>
<td>20.81↓</td>
<td>23.12</td>
<td>32.4</td>
</tr>
<tr>
<td>AST-U/l</td>
<td>122-321</td>
<td>128.4</td>
<td>131.28</td>
<td>151.4</td>
</tr>
<tr>
<td>GLU - mg/dl</td>
<td>54-93</td>
<td>48.81↓</td>
<td>55.26</td>
<td>56.85</td>
</tr>
<tr>
<td>LDH-U/l</td>
<td>811-1250</td>
<td>858.3</td>
<td>1254.98↑</td>
<td>1183.33</td>
</tr>
<tr>
<td>GGT-U/l</td>
<td>60-101</td>
<td>60.54</td>
<td>88.52</td>
<td>90.81</td>
</tr>
<tr>
<td>TRIG - mg/dl</td>
<td>10-29</td>
<td>12.2</td>
<td>18.29</td>
<td>22.6</td>
</tr>
<tr>
<td>CK-U/l</td>
<td>28-130</td>
<td>52.71</td>
<td>87.98</td>
<td>91.23</td>
</tr>
</tbody>
</table>
As it can be noticed from the data in the table, there are some minor blood biochemical panel changes (increasing of CREA, TP and ALB), without clinical significance, probably caused by the high protein diets.

Conclusions

Following the study, the welfare level in the assessed goat farm was found acceptable. However, further improvements addressing the noticed deficiencies (light intensity, lack of outdoor access) can result in an optimum welfare level.

Acknowledgements

This study was conducted within the Postdoctoral research project Farm animals’ welfare in relation with national and European provisions in the perspective of echo-sano-genesy, Animal Science Biodiversity and Food Biotechnologies Postdoctoral School, Romanian Academy – National Institute of Economic Research Costin C. Kiritescu, HRDSOP 2007-2013

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SKULL MORPHOLOGY IN COUGAR (*Puma concolor* - Linnaeus, 1771) - CASE STUDY

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**Summary**

The cougar (*Puma concolor*) is included on the Red List of the International Union for Conservation of Nature (IUCN Red List) as least concern species; in Romania being present only in zoological gardens. Thus, we have considered that the skull morphology description of this species is useful for veterinarians that ensure the health of animals in zoos. Also, we consider the report useful for the customs agents because there can be cases of feline trophies legal or illegal trafficking, the specific features of the skull offering the differences between species less vulnerable as cougar (*Puma concolor*) and strictly protected species such as the leopard (*Panthera pardus*) or cheetah (*Acinonyx jubatus*), these two species having the dimensions close to those of the cougar. For the study, we have used a skull from an adult cougar, aged 18, female, died of natural causes and donated to the Anatomy Department, Bucharest Faculty of Veterinary Medicine for teaching purposes by the Bucharest Zoo. The subspecies to which this individual belongs is unknown. The skull was obtained after removal of soft tissue and after a controlled soaking process, it was washed and degreased. The skull description was made in accordance with the *Nomina Anatomica Veterinaria*, 2005. The skull is characterized by a small-sized viscerocranium, with a compact brain case. The cranial base is short but wide. The pterygoid hook is very long, sharp, aborally oriented. The cranial cavity is highly developed, representing nearly 2/3 of the middle section of the head bone. In conclusion, the studied skull ranged with most features within the ones belonging to the domestic cat, according to the specific literature.

**Key words**: cougar, skull, anatomical features

Cat bones and especially skulls often appear as evidence in forensic cases on wildlife (especially in the United States and in most European countries) in case of imports of trophies from more or less endangered species such as: cheetah, jaguar, leopard, puma, snow leopard/irbis, lion and tiger. Due to the fact that these species have different degrees of protection in international law, it is necessary an exhaustive examination to identify the exact species. Comparison of skeletal morphology is a classical method to study the degree of similarity between species, especially for the taxonomic classification and differentiation.
The skull dimensions of large felids partially overlap and can be hard to identify, especially when it is not known the geographical area of origin. Articles about taxonomy and systematic (phylogenetic relations) of cats contain little information about comparative morphology, especially the skull (Werdein, 1985; Garcia-Perea, 1994). Angela von den Driesch (1976) designed and described the standard measures of cat skulls, especially those found in archaeological sites (2,3,4). Seymour (1999) compared the skull morphology of small feline from South America and added some features for some species (2,3,4). The small wild cats morphology was described by Lekagul and McNeely, 1988; de Oliveira, 1998; Garcia-Perea, 2002 (5,6,11). The skull morphology of cats from North America was described by Currier, 1983; Tumlison, 1987; Seymour, 1989; Lariviere Walton, 1997; Murray and Gardner, 1997; de Oliveira, 1998 etc. (4,5,6,10,11). Skull morphology of medium and large feline of the genus *Panthera* was partially described by Todd, 1966; Werdelin, 1983; Lamerichs, 1985; Seymour, 1989; Larsen, 1997 (1,7,8,9,12,13,14,15).

In Romania there are few and disparate data regarding the description of skeleton of some species of cats found in zoos or circuses menagerie (5,6). The main objective of this study was to provide useful information for veterinarians working in zoos, custom officers and other professionals interested in tools of morphological recognition (13,17,18,19,20), based on the particularities of the skull for endangered species or subjected to legal or ilegal trafficking. Also, it was aimed to obtain a representative iconographic material in order to facilitate the understanding of the obtained morphology aspects.

**Material and methods**

In order to describe the morphology of the skull was used the skull of an adult cougar, 18 years old, female, donated for teaching purposes by Bucharest Baneasa Zoo to the Anatomy Department from the Faculty of Veterinary Medicine of Bucharest. The animal died from natural causes (old age). It should be noted that cougar is an exotic animal and can be studied only in very rare cases in Romania.

The skull was obtained after removal of soft tissue, then subjected to controlled soaking process, washed and degreased. Deep cleaning was achieved by the removal of all soft formations on the surface of the bone. Maceration was carried out at a constant temperature for a long time (about 50 days) under continuous surveillance. Washing was carried out in a first step in running water for 24-48 hours.

Re-cleaning after maceration was carried out with the help of the tip of the scalpel to remove any organic residues. Scouring was carried out using the diluted cleaning detergents in the wash water and after washing with slightly acid water (adding 10% acetic acid) to verify the removal of any trace of organic matter. Drying was carried out under surveillance for 48-56 hours at an average
temperature of 18-22°C to prevent cracking bone structures. Whitening of bones was carried out by immersing the skull in hydrogen peroxide solution (H₂O₂) 30% and then drying.

**Results and discussions**

The dorsal side of the skull of cougar (*Puma concolor*). The skull is characterized by a small-sized viscerocranium, with a compact brain case. The dorsal surface of the skull is relatively flat and elongated in the torches. The exocranial face of the parietal is convex in all directions (fig. 1).

![Skull of a cougar (Puma concolor) – dorsal side (original)](image)

Fig. 1. Skull of a cougar (*Puma concolor*) – dorsal side (original)

The occipital protuberance is high and well highlighted and nuchal ridges are well developed, with a maximum height in the rear plan. External sagittal crest, which has the maximum height at the interparietal level, is not very high (cause the animal is a female). At frontal level, external sagittal crest is divided into two temporal lines that extend to the free end of the frontal zygomatic process. Zygomatic processes of the frontal part are relatively long and latero-ventral-aboral orientated. Nazals are slightly elongated, their lateral edges ending in a pointed front in the rostral end, attached to the medial edge of the incisor. The relief of canine socket is less obvious.

The lateral side of the skull of cougar (*Puma concolor*). The lateral side of the skull has an obvious zygomatic arch with a lateral convexity and an obvious zygomatic process (Fig. 2). Temporal fossa is reduced in depth and the relatively large. In the auricular region there is an obvious ear tympanic bulla, elongated and convex in every direction. The external auditory canal, with the appearance of large hole, relatively oval, is situated latero-rostral from the tympanic bulla.

The mastoid process is reduced and a flanked at base by a stilo-mastoid foramen. The styloid process is missing and the paracondilar process is separated from the mastoid process by a low notch. The orbit is large, with an incomplete contour aboral. Orbito-temporal ridge is very low. The orbit has a very broad communication with pterigo-palatine fossa, at which level there is a foramen in the mandible, a very reduce palatine foreman at aboral level, and medio-aboral is present an obvious sfen-opalatin foramen with oval aspect.

On the anterior outline of the orbit above the second molar of the maxilla, there is an obvious infraorbital foramen. The fossa of lacrimal sac is slightly visible, with a foramen in the middle. On the anterior part of the orbit in can be seen, almost on the half, one lacrimal tuber.
Fig. 2. Skull of a cougar (*Puma concolor*) – lateral side (original)


Orbital hiatus is shallow, having the following openings: ethmoid foramen, the optical foramen, the round foramen and the orbital fissure, lacking the alar channel and foremen. Vento-aboral from the round foramen is observed an oval foramen. The base of the presfenoid wings is passed through the optical channel which opens though the optic foramen above the optic orbital foramen.

The ventral side of the skull of the cougar (*Puma concolor*). As the vast majority of cats, the skull base is short, but wide. The occipital foramen is flanked on both sides by one condyle with a slightly oblique arrangement from top to bottom, and the joint surface is convex from top to bottom. The muscle tubers from the exocranian front of the bazioccipital can be slightly observed. At the base of the tympanic bulla, mid-aboral is seen the jugular foramen (fig. 3).
On the aboral part of the jugular foreman it can be seen the hypoglossal nerve canal opening. Jugular processes are reduced and arranged at the end of the tympanic bulla, which it covers completely. In the rostral end of the tympanic bulla two openings can be observed: revealed two openings: medio-rostral - the spinos foramen and lateral from this one - the carotid foramen.

At the level of the zygomatic process of the temporal are present a glenoid cavity, narrow and transverse elongated and one retroarticular process, widened and rostro-ventral orientated. The hamulus of pterygoid (*Hamulus pterygoideus*) is very long, sharp, aboral oriented.

![Fig. 3. Skull of a cougar (*Puma concolor*) – ventral side (original)](image)


In the rostral extremity, it can be observed the palatine fissures with ellipsoidal feature which continues in rostral direction for the internal face of the incisor bone with obvious incisor fossa. The vomer is highly developed, with narrow
aboral end and the rostral widened, ending at the rostral end of the two nasals. Due to the narrowing of the aboral end, the area of communication between the two nasal cavities is relatively small. Anterior palatine foramen are reduced and continue in rostral direction with slightly visible palatine fossa.

Nuchal side (aboral side). Nuchal ridges are visible and occipital protuberance is reduced. Jugular processes are reduced and ventral-aboral oriented. Condylar fossa are reduced and the occipital foramen is relatively large and rectangular.

Cranial cavity of cougar (*Puma concolor*) is highly developed, representing almost 2/3 of the middle section of the head bone. The tentoric process (*processus tentoricus*) is highly developed, fully attached to the parietals, which makes the cerebrum and cerebellum parts to be completely separated in superior plan.

Nasal cavities of cougar (*Puma concolor*). The two aboral halves of the nasal cavities communicate one with other through the nasopharynx fossa, which bone part represents the aboral half of the floor. Ethmoid volutes, highly developed, are present mostly in the nasal cavity thus the ventral nasal conches, with a layered structure, are found only in the rostral half of the nasal cavity.

The mandible of cougar (*Puma concolor*) is an even bone. The ventral edge of the horizontal portion of the mandible is straight. Angular process, very developed, is easily controlled rear. Maseterin fossa is deep and wide, reaching halfway up to coronoid process. In cougar can be observed a mental foramen and an accessory (lateral) mental foramen. Condilian process has relatively cylindroid aspect, with convex articular surface. Coronoid process is recurved aboral and free edge is rounded.

Linear measurements were performed to calculate the facial and cephalic index of the skull. Facial index was calculated using the formula - width of the face (3.8) x 100 / length of the face (ln_1 - 4.3). The facial index of the puma from this study is 88.37, thus the viscerocranium surface is short (Face index = 3.8x 100 / 4.3 = 88.37%). Cephalic index was calculated according to the formula - the width of the zygomatic arch (zw - 13.4) x 100 / the maximum length of the skull (GSL - 18.6). The puma cephalic index value is 63.86%. Due to this result, this puma is considered a mammalian with very large head bone, greater than 46% (cephalic index 13.4x = 100 / 18.6 = 72.04%).

**Conclusions**

The skull is characterized by a small-sized viscerocranium, with a compact brain case.

The dorsal side of the skull is elongated and relatively flat from the torches. Occipital protuberance is high and well highlighted and nuchal ridges are well developed, with a maximum height in the rear plan. The frontal zygomatic processes are relatively long, the latero-ventral-aboral routed.
The lateral side presents an obvious zygomatic arch, with the convexity placed lateral the with the zygomatic process well shown. In the auricular region is observed a clear tympanic bubble elongated and convex in all direction.

Disposed rostral lateral from the tympanic bubble, external auditory canal is observed, with the appearance of large hole, relatively oval. The styloid process is missing and the paracondilar process is separated from the mastoidian process by a notch. On the edge of the orbit neat to its half, a lacrimal tuber is present.

At cougar (Puma concolor) the base of the skull is short but wide. Occipital foreman is flanked on both sides by one condyle with a slightly oblique arrangement from top to bottom, and the joint surface is convex from top to bottom. The muscle tubers on the exocranial face of the bazioccipital are slowly shown. In the rostral end of the tympanic bubble revealed two openings: thorny foremen rostral medial and lateral the foremen carota. Pterigoidian hook is very long, sharp, aboral oriented. Anterior palatine foremen are reduced and continue in rostral direction with slightly visible palatine fossa.

The nuchal ridges are visible and occipital protuberance is less developed. Jugular processes are reduced and ventral-aboral oriented. Septic condylar are reduced, and the occipital foreman is relatively large and rectangular.

The two aboral halves of the nasal cavities communicate one with other through the nasopharynx fossa, which bone part represents the aboral half of the floor. Ethmoid volutes, highly developed, are present mostly in the nasal cavity thus the ventral nasal conches, with a layered structure, are found only in the rostral half of the nasal cavity.

The mandible is an even bone. The ventral edge of the horizontal portion of the mandible is straight. Angular process, very developed, is easily controlled rear. Maseterin fossa is deep and wide, reaching halfway up to coronoid process. In cougar can be observed a mental foramen and an accessory (lateral) mental foramen. Condilian process has relatively cylindroid aspect, with convex articular surface. Coronoid process is recurved aboral and free edge is rounded.

The face index of cougar (Puma concolor) is 88.37, therefore the viscerocranium surface is short.

The cephalic index of cougar (Puma concolor) has a value of 72.04. Due to this result, this puma is considered a mammalian with very large head bone, greater than 46%m this skull having a spheroidal shape.

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Assessment of Red Wine Quality Parameters in Correlation with Consumers' Safety

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Summary

In the context of high consumption of different types of wine and given the consumer demand regarding food safety, the purpose of this study was represented by the quality control of these products using physicochemical methods. The aim of this study was to perform a comparison between 3 types of red wines quality parameters and the legislative requirements. Twelve samples of red wine (2 types of dry wine – Merlot, Cabernet Sauvignon and one type of semidry wine - Black Feteasca) were submitted to analyse regarding the alcoholic concentration, total acidity, total sulphur dioxide, unreducible dry extract and residual sugar. The results ranged from 12.5 to 13.8 % vol for alcoholic concentration, 5.04 to 6.52 g tartaric acid/l for total acidity, 85.4 to 176.9 mg/l for total sulphur dioxide, 22.6 to 24.59 g/l for unreducible dry extract and 0.96 to 9.6 g/l for residual sugar. All red wine assortments respected the product specifications imposed by the legislation and producer.

Key words: red wine, physicochemical methods, consumers' safety

The diversity of natural climatic conditions, assortments, preparation technology and taste determine the existence of a very large range of wines, which differ in terms of chemical composition (1, 5).

The major factor involved in wine quality is the composition of grape berries at the time of the harvest. So, grape quality is also referred to as wine quality potential (6). Changes during the berry ripening affect the composition of the future wines regarding the sugar content, aromatic compounds and colour (4).

Taking into consideration the high consumption of different types of wine and given the consumer demand regarding food safety, the purpose of this study was represented by the quality control of these products using physicochemical methods.

Materials and methods

Twelve samples of red wine (2 types of dry wine – Merlot, Cabernet Sauvignon and one type of semidry wine - Black Feteasca) were collected from a medium sized winery from the south part of Romania. Using standardized methods, these samples were submitted to analyse regarding the alcoholic concentration, acidity, total sulphur dioxide, unreducible dry extract and residual sugar.
Results and discussions

The results were presented for each type of wine (Merlot, Cabernet Sauvignon and Black Feteasca) and each type of analyze.

Results of quality parameters assessment Merlot type

The results ranged from 12.8 to 13.6 % vol for alcoholic concentration, with an average of 13.3 % vol (Fig. 1).

![Fig. 1. Results for alcoholic concentration (% vol)](image1)

For acidity, the results ranged from 5.07 to 6.25 g tartric acid/l, with an average of 5.5g tartric acid/l (Fig. 2).

![Fig. 2. Results for acidity (g tartric acid/l)](image2)

The results ranged from 85.4 to 158.2 mg/l total dioxide sulphur, with an average of 118.5 mg/l (Fig. 3).

![Fig. 3. Results for dioxide sulphur (mg/l)](image3)
For unreducible dry extract content, the results ranged from 23.18 to 24.12 g/l, with an average of 23.45 g/l (Fig. 4).

The results ranged from 0.9 to 1.2 mg/l residual sugar, with an average of 1.05 mg/l (Fig. 5).
The results of the physico-chemical analyzes are in ranges imposed by legislation. The alcoholic concentration should be between 8.5-14%, with an average value obtained of 13.3%. The total acidity has an average value of 5.5 g acid tartric/l compared to the reference value of at least 3g acid tartric/l. The total SO₂ concentration has an average value of 118.5 mg/l, being below the maximum limit of 160 mg/l. The unreducible dry extract content has a value of 23.45 g/l, compared to the limits of 18-28 g/l. Being a dry wine, reducing sugars are below the limit of 4g/l, with an average value of 1.05g/l (2,3).

Results of quality parameters assessment Cabernet Sauvignon type

The results ranged from 12.5 to 13.8 % vol for alcoholic concentration, with an average of 13.25 % vol (Fig. 6).

![Fig. 6. Results for alcoholic concentration (% vol)](image_url)

For acidity, the results ranged from 4.95 to 6.17 g tartric acid/l, with an average of 5.39 g tartric acid/l (Fig. 7).

![Fig. 7. Results for acidity (g tartric acid/l)](image_url)

The results ranged from 97.8 to 176.9 mg/l total dioxide sulphur, with an average of 142.55mg/l (Fig. 8).
For unreducible dry extract content, the results ranged from 23.15 to 24.59 g/l, with an average of 23.69 g/l (Fig. 9).

The results ranged from 1.3 to 2.1 mg/l residual sugar, with an average of 1.5 mg/l (Fig. 10).

Alcoholic concentration should be between 8.5-14%, with an average value of 13.25%. The total acidity has an average value of 5.39 g tartaric acid / l compared
to the reference value of at least 3g tartaric acid/ l. The total SO₂ concentration has an average value of 142.55 mg / l, being below the limit of 160 mg / l. The non-reducing extract has the value of 23.69 g / l, ranging from 18-28 g / l. Being a dry wine, reducing sugars are below the limit of 4g/ l, with an average value of 1.5g/ l (2,3).

Results of quality parameters assessment Black Feteasca type

The results ranged from 12.2 to 13.8 % vol for alcoholic concentration, with an average of 13.05 % vol (Fig. 11).

Fig. 11. Results for alcoholic concentration (% vol)

For acidity, the results ranged from 5.04 to 5.96 g tartric acid/l, with an average of 5.61 g tartric acid/l (Fig. 12).

Fig. 12. Results for acidity (g tartric acid/l)

The results ranged from 93.6 to 164.3 mg/l total dioxide sulphur, with an average of 113.5 mg/l (Fig. 13).
For unreducible dry extract content, the results ranged from 22.60 to 23.51 g/l, with an average of 23.02 g/l (Fig. 14).

The results ranged from 7.3 to 9.6 mg/l residual sugar, with an average of 8.55 mg/l (Fig. 15).

For Black Fetească, all results of the physico-chemical analyzes are in ranges imposed by legislation. The alcoholic concentration should be between 8.5-14%, with an average value of 13.05%. The total acidity has an average value of 5.61 g / l compared to the reference value of at least 3g / l. The total SO₂ concentration has an average value of 113.5 mg / l, being below the maximum limit.
of 160 mg/l. The non-reducing extract has the value of 23.02 g/l, between the limits of 18-28 g/l. Being a semidry wine, the reducing sugars have reference value between 4-12 g/l, the obtained average value being 8.55 g/l (2,3).

Conclusions

In the studied vinery the technological flow for red wines respects the technological flow encountered in most of the specialized units, with small variations that give the originality of the products.

The data analysis shows that there are no significant differences between the three red wine varieties analyzed.

In conclusion, we can say that the products obtained within the studied unit meet the quality requirements imposed by the legislation and the consumption of these products does not represent any risk for consumers’ health.

References

EVALUATION OF COLOSTRUM CELL CONTENT 
AND BUFFALO MILK

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Summary
In this study, some investigations were performed regarding the evaluation of the cellular content of the colostrum samples (n=5) and buffalo milk (n=15), using the automatic method (Somacount system) and the microscopic one (Milk cytogram). Milk cytogram testing was performed on colostrum and milk’s sediment smears (obtained by centrifuging the milk), Panoptic colored. Cellular structures from the colored smears were identified and characterized based on morphological aspects, states of activity and dyeing affinity. As expected, SCC values were higher (87.5x10³/mL) in colostrum than in milk (36.7x10³/mL). In the two samples selected from the day of calving colostrum, SCC determination was not possible due to the high cellular content of this, as revealed and assessment of smear (+++ / ++++). The overall evolution of SCC revealed a relatively tight score, but with lower values for milk than colostrum. Analysis of milk cytogram revealed the possibility of reducing the quality of smears by a rich fat content in the buffalo milk. In addition to this, we observed a high quality of smears prepared from the milk sediment washing compared to undiluted. We obtained good quality smears degreased by immersion in organic solvents. Milk cytogram evolution revealed a case with an elevated frequency of PMN leukocytes (32.5%), achieving a double average rate. This growth was achieved due to lower lymphocytes. It should be noted, however, the increased proportion of lymphocytes in 5 cases, exceeding 30%. Milk cytogram configuration and the relation between the indicated cell types or certain cell adherence to other structures were determined, resulting in the formation of micro-conglomerates intercellular adhesions. Finally, the milk cytological overall data has been completed with the frequency of atypical microbial cells. In conclusion, we mention that the dominant cytological buffalo milk can be considered the lymphocyte population growth trend, without association with increased microbial load.

Key words: somatic cells number, cytograms, milk, colostrum, buffalo cow

The bubalines raised in households or specialized farms represent an important alternative milk source, in Romania as well as in many other countries. Thus we consider as relevant the data from 1999, which show that from the global milk production of 564 million tones, 63 million tones belong to buffalo milk (3,12, 13, 14).
Materials and methods

Dairy secretion samples for cytological investigations were harvested from 20 healthy buffaloes. The samples were prevailed during the months of April - May, when some buffaloes were in the lactation phase and others in full lactation. As a result, we managed to form two batches according to the lactation stage. Lot 1, consisting of 15 buffaloes in full lactation, without clinical changes of the mother or organoleptic milk. The reactions to the Californian test were negative for the secretions collected from each quarter of the buffalo mammal in this lot. Lot 2 consisted of five buffaloes in the colostrum period, two of which were in lactating onset (first and second day). The health of the mammary gland was appreciated during this period based on the negative results of the clinical examination and the organoleptic examination of the colostrum carried out by the Contrast test.

Hygienisation and disinfection of the mammary glands required the preparation of hot water bottles, individual gauze towels, and conical alcohol glasses, and sterile and individualized glass tubes for the collection of milk secretion samples. The examination of the first milk jets was performed by the R-Mastitest, with bromcresol blue reagent. In the case of colostrum, the organoleptic examination was carried out by the Contrast test, based on the use of a black photo trays, which revealed the most discreet changes in colostral secretion. The harvesting procedure consisted of a nipple disinfection, followed by sampling sterile tubes, previously labeled. As soon as the samples were harvested, they were placed on ice and immediately transported to the laboratory (3).

The determination of the total somatic cell count in milk (SCC) was performed with the SOMACOUNT (Bentley-USA) device, and the processing of the mammary secretion samples in the form of smears required: degreased histological blades, centrifuges, pipettes, degreasing solutions Methyl acetate and acetic acid), MGG dye battery. Comparatively, Dia Panoptic Quik staining with three baths, including: fixation and pre-coloring solution, eosinophil solution and base solution, was also used (8).

Results and discussions

The surveys conducted in Salaj County, an area of great tradition in the production of buffalo milk, revealed an extremely low frequency of mammary diseases in this species. Moreover, the data reported by the Sanitary Veterinary Service and the owners revealed that in the 9 buffaloes no mastitis or other mammary diseases were reported from the beginning of the first lactation until the present. Taking everything into consideration, this reveals a good natural buffaloes resistance to mastitis, despite the poor zoo-hygienic conditions in which they are maintained.

The SCC determination is currently a test of major importance for animal health and public health surveillance, with legal implications. However, the regulations regarding the SCC in goat milk, and particularly, sheep or buffalo milk,
are not yet standardized, the automatic counting method is already widespread in goat farms and milk processing centers of this species. For the control of buffalo milk the method is rarely used. In fact, research in this regard is quite sporadic, with few data being so confusing (9).

Our research, motivated by the above considerations, revealed by the individual data from table no. 1 variation in relatively limited limits of SCC in a group of 19 buffalo animals in the grazing season. As shown in this table, the SCC values (cases 14-17) were higher (87.5x10³/mL) than those in milk (36.7x10³/mL).

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<td>18</td>
<td>182.4</td>
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<tr>
<td>19</td>
<td>−</td>
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<tr>
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<td>−</td>
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<td>+++</td>
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<tr>
<td>Media</td>
<td>48.72</td>
<td>++</td>
<td>++</td>
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</tbody>
</table>

Moreover, in the case of the first colostrum (samples 18 and 19), dosing was not possible because this secretion is hyper concentrated as it results from the assessment of cellular content on smear (+++ and ++++ respectively). Based on individual data, an average of 48.7x10³ cells/mL of milk was determined, the investigated sample including 17 buffaloes and 68 mammary quarters.
The data set in this table also shows the correlated evolution of SCC with the non-frail cellular structures in the milk cytogram structure (atypical cells and microbial cells). In this regard it can be seen that increased SCC samples usually contain more atypical and microbial cells (cases 2 and 9). It is also easy to see the direct proportional correlation between SCC and the level of cellular content assessed on smear (cases 9, 14, 16, 17). There have also been some exceptions to the above mentioned dominant trends, as is the case for Samples no. 1 and 8, which had low SCC and increased cellular content on smear. The overall evolution of SCC individual values in investigated buffaloes is clearly illustrated in the graph of Figure 1, showing a relatively low, but lower value for milk than colostrums.

Fig. 1. The evolution of SCC in buffalo milk studied

SCC also presented variations in the health status of buffaloes studied. Without much data in this regard, we report in Table 2 a statistic showing SCC growth over 100x10^3/mL for 3 endometritis or non-nephrotic buffaloes compared to healthy animals where the SCC level did not exceed the limit of 64x10^3/mL. These are two cases with endometritis at which SCC had a value of 108.6-124.3x10^3/mL, and a nephrosis case where the SCC level was 182.4x10^3/mL.

The quality of the preparations was also ensured by the continuous degreasing of the used ustensils and the additional degreasing of the smears in organic solvents.

The relevance of the lactocytogram performed on the 19 samples investigated was based on the inclusion in the microscopic examination of a representative surface of smear with the identification of at least 200 cellular structures. In order to identify and classify the cell types and subtypes, we used morphophysiological criteria with good results. The morphological criteria were based mainly on the appearance of the nucleus: shape, volume, degree of segmentation, chromatic material distribution, presence of nuclear nucleus or nuclear corpuscle. In the analytical analysis of the criteria used, we attributed the major impact to the cytoplasmic characteristics with the interpretation of the activity...
states corresponding to each evolutionary phase, evaluating the nucleus/cytoplasm ratio, cytoplasmic volume and tincture (1, 5).

Of all the examined samples, eosinophils were found in 9 cases, representing 47.36% of the stock. The mean value of 0.7% revealed the low frequency of this leukocyte subcategory in buffalo milk. At the same time, the tight limits of variation of individual values are registered, with the upper share rising to only 2% and only one case. From all the results obtained, lymphocytosis did not exceed in any of the cases the values of 35-40%, which are the maximum limits recorded in the cow. In 3 cases, values below the limit of 10% (5.6 - 8.5%) were observed, considered by some researchers in the field as minimal threshold. The morphological particularities related to the states of activity encountered in the monocyte - macrophage populations have allowed us to achieve a subclass of this category. Thus, due to their enhanced polymorphism, mononuclear phagocytes were subdivided into three sub-categories: monocytes - inactive macrophages, active macrophages and hyperactive macrophages. The analysis of the correlated evolution of inactive, active and overactive macrophages results in their distribution in a ratio of approximately 3: 2: 1. This report presents more or less significant oscillations, with predominance of inactive macrophages in 5 cases, those active in 2 cases, and overly hyperactive 2 cases. Noteworthy is that for colostrum the ratio recalls 1: 2: 3, that is, the opposite of the same as for milk. Recognition and identification of epitheliocytes was mainly based on the particular form of the cell, which retains the tendency to cuboidal or even semi-high cell appearance, with the eccentric nucleus in the advanced state of cariopicnosis.

Most of the epitheliocytes signaled in the colostrum or milk sediment showed the above-mentioned features in terms of cell shape and nucleus morphology. These were completed by a strongly basophilic and well-represented cytoplasmic mass, sometimes spherical or ring-shaped, due to intracytoplasmic corporal fat spheres. All of these morphophysiological characteristics correspond to the alveolar cells, also known as the Lactocytes. In some smears prepared from the "washed" lactosediment, we highlighted cell structures with atypical morphology, whose origin could not be specified and consequently could not be identified. In this category, we have introduced only those formations whose nucleus has been highlighted without integrating them into the lactocytogram configuration, being recorded using conventional symbols.

Although an enormous amount of data has accumulated in the area of lactocytology, there is still discussion and controversy over the morphophysiological characteristics and especially the origin of the milk cell populations. These uncertainties make us consider inappropriate some variants of expression of the notion of milk cells themselves. Thus, many researchers reunite all these structures under the name of somatic cell count (SCC) in milk, although not all of them have somatic origin. In order to respond to their fairly diverse origins, we consider the name of the total number of cells in milk more appropriate (7, 9,10).
Conclusions

The total number of dairy cells is a consistent indicator of the physiological and pathophysiological changes of the mammary gland, the individual determinations being used for mammalian health surveillance, and the ones used on the collection milk to monitor the evolution of mastitis in the actual and implicitly hygienic quality of the milk.

The current SCC standards are rigorously respected in the case of cow's milk and are outlined for the goat, which also gives them legal implications for public health. In the context of those presented above, the results of this study are advancing relevant SCC values (48.72x10^3 cel./mL) and the proportion of cell types in buffalo milk (16.4% PMN, 25% lymphocytes, 55.5% 2.6% epithelial cells).

References

SURGICAL MANAGEMENT OF IATROPATIC URETHRA RESECTION IN A BITCH: A CASE REPORT

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Summary
The urethral injury in female dog is a rare condition. This report describes a urethral injury in a bitch by iatrogenic surgical resection of the urethra after vaginal hyperplasia surgery and subsequent treatment in Surgery clinic of the Faculty of Veterinary Medicine Timisoara where was applied a similar technique with that used in female patients, namely vaginourethroplasty with urethral reconstruction using vaginal flap. For 45 days after surgery the animal showed signs of urinary incontinence without any signs of ascending urinary infections. At recheck performed at 90 days the owner tells that the animal adopt the miction position urinating in continuous flow. The animal shows a favourable evolution after surgical intervention at time (six months) when this paper was submitted for publication.

Key words: dog, iatrogenic urethral injury, urethral reconstruction

The urethral injury in female dog is a rare condition seen by veterinarians. As urethral rupture in female dog has been reported uncommon compared with male (13). The most common cause of urethral rupture in dogs was motor vehicle trauma with pelvic displacement and fractures (1, 6, 11, 15), bite wounds (15) and iatrogenic catheterizations (13). Iatrogenic urethral injury may occur when surgical dissection near the urethra must be done (5).

Treatment options include temporary urinary diversion through cystostomy or urethral catheterization and second intention healing, permanent bypassing of the traumatized area through urethrostomy proximal to the injury site, primary repair of transection injuries (9), and urinary diversion procedures (7) by uretero-colonic, uretero-jejunum, uretero-ileal anastomosis (11) or by reimplantation of the ureters in the proximal aspect of the vagina (10, 19).

This report describes a management of total urethral resection in a bitch after vaginal hiperplasia surgery by vaginourethroplasty with urethral reconstruction using vaginal flap.

Case description
A one-year-old, intact female, Central Asian Sheppard dog, weighing 62 kg, was referred to the Surgery clinic of the Faculty of Veterinary Medicine Timisoara for acute urinary retention (excretory anuria) lasting of ten days.
Prior to admission with 10 days ago the animal was subjected in another service, reproduction, a resection surgery of vaginal hyperplasia. With 24 hours ago it was mounted, in another service, an urethral catheter and were collected blood samples for biochemical analysis. Anamnestic data reveal: a continuous deterioration of general condition, sadness), lack of spontaneous locomotion, loss of apetite, repeated vomiting, bloating, absence of urination. In the first two days after surgical resection of the prolapsed vaginal mass (vaginal hyperplasia) was observed a difficult urination in drop, and then the discharge of urine has completely cease. Gentamicin was given to the animal.

After admission the dog was clinically examined. The following were noted: animal adopt the urination position without micturion, difficult walk, pronounced preference for decubital position, body temperature of 38 °C, heart rate 118 beats/minute, respiratory rate 36 breaths/minute, sadness, persistent cutanate fold – mild dehydration, arterial pressure 178/149(162) mmHg, distended abdomen, globe bladder, abdominal contracture at palpation, lack of operatory wound corresponding to episiotomy, healed surgical vaginal wound without visible traces, urinary meatus having the aspect of infiltrative swelling, the presence of a urinary catheter (black arrow) fixed throught vaginal mucosa, on lateral side of the urinary meatus (UM) – fig 1.

Blood analyzes showed the following changes from normal: MCH -24.9 pg, WBC - 19.230 mm³, TGO (ASAT) – 61 U L, TGP (AALT) – 41 U/L, nitrogen ureea - 130.1 mg/dL, serum creatinine 2.98 mg/dL and a normal value for serum potassium of 4.7 mmol/L.

The attempt to urethral meatus catheterization failed, catheter advancement into the urethra beeing possible only for about 2 cm.
Fluoroscopic examination (Siemens Siremobil compact L) of the abdominal cavity reveals the existence of a strongly dilated bladder and the image of urinary catheter mounted before the patient’s admission in Surgery Department, crossing the vaginal mucosa, on lateral side of urinary meatus, shows that it does not get into the urinary bladder (black arrow), its end being found in the abdominal cavity (white arrow) – fig 2.

The urography with contrast material (iodine 275 mg) using the catheter placed one cm into the urinary meatus show non-existence of an urethral conduct (black arrow) and diffuse outpouring of the contrast agent into pelvic cavity tissues (white arrow) and on the X-ray image appears the path of the catheter fitted through vaginal mucosa, on lateral side to the urinary meatus (double arrow) – fig 3.

The urethroscopy of urinary meatus (Hopkins forward-oblique 30°, 3.5 mm diameter, 36 cm working length - Karl Storz GmbH Germany) has revealed a short urethral conduct (about 10 mm), totally blocked by the presence of a suture thread.

After emptying through cystocentesis of a large quantities of urine, 3200 ml, it was decided to continue the investigations using laparoscopic devices.

Premedication with diazepam (Diazepam 0.5%, Terapia SA, Romania) (0.4 mg/kg b.w., IV) and ketamine (Ketamin 10%, CP-Pharma, Germany) (5 mg/kg b.w., IV) was followed by induction with propofol (Norofol 1%, Norbrook Laboratory Ltd., North Ireland) (3 mg/kg b.w., IV) and then was performed endotracheal intubation. General anaesthesia was maintained with isoflurane vaporized in oxygen and intermittent positive pressure ventilation was used. After the routine preparation of the ventral abdominal wall and animal’s immobilization in dorsal decubitus, Trendelenburg position - 15° inclination, was held the laparoscopy of abdomeno-pelvic cavity.
The trocar-cannula (6 mm diameter with multifunctional valve - Karl Storz GmbH Germany), was inserted subombilical and the abdominal cavity was insufflated with CO₂ (10 mmHg). The trocar (upon cannula with multifunctional valve) was replaced by the laparoscope (Hopkins, forward 0°/ 5 mm diameter/ 29 cm length - Karl Storz GmbH Germany) and the caudal portion of the abdomen was examined.

The examination of the abdominal cavity revealed a relaxed urinary bladder (UB) with a sealed bladder neck by a suture wire (white arrow), non-existence of a continuity with uretra (black arrow), and the inability to detect a possible uretral stump – fig 4.

![Fig. 4. Laparoscopic image of abdominal cavity](image)

Following these findings and the fact that the owner refused two proposed therapeutic options: permanent urine deviation by colonic repositioning of ureters (ureterocolic anastomosis) and a ventral approach to the pelvic canal by bilateral pubic and ischial osteotomy for urethra reconstruction, it was decided the open approach of abdominal cavity through omblilico-pubic celiotomy omblilico-pubian and concomitantly of the vagina by a large episiotomy.

The surgical procedure consisted of the urethral reconstruction using a vaginal mucous membrane flap, being a similar technique with that applied in female patients (8).

The urinary bladder was completely emptied by puncture, the suture wire placed on bladders neck was removed. From dorsal vagina wall, from the canula located above, was constructed by incision and dissection a mucous membrane and muscle flap, having a length of about 4 cm, which was sutured in a tube-like shape on the metallic cannula of 6 mm. Booth, metallic cannula with flap prepared as tube-like shape were inserted instead of the catheter placed through the vaginal mucosa, on the lateral side of urinary meatus, and were protruded inside the abdominal cavity, the end being then placed inside the bladder. Anastomosis between the bladder neck and new urethral path (vaginal mucou-muscular flap prepared as tube) was realised in simple interrupted penetrating suture using 2
metric poldioxanone – PDS (PDO BioSintex Romania). Place the donor flap used for reconstruction was sutured in continuous inverted suture type using the same suture material (black arrow). Flap’s vaginal end was fixed (in simple interrupted penetrating suture with 2 metric poldioxanone – PDS (PDO BioSintex Romania) in a circular arrangement to the vaginal mucosa situated around the cannula (white arrow), dorsal to the urinary meathus – fig 5.

![Image](image.png)

**Fig. 5.** Final image of urethral reconstruction

The neourethra was constructed from the dorso-lateral vaginal wall. Later metal cannula was replaced with a urinary catheter secured through suture to vulvar labia.

The routine closure of the celiotomy was performed. The episiotomy site was closed routinely in three layers (16). Vaginal mucosa was apposed with 2 metric polyglycolic acid – PGA (Sentesorb) using interrupted sutures. The muscular layer was sutured in a continuous pattern using 2 metric polyglycolic acid – PGA (Sentesorb). A simple continuous pattern with 3 metric nylon - Monolon (Sentesorb) was used to close the cutaneous wound.

The urinary catheter was connected to a urine collection bag with bottom outlet and was maintained for ten days postoperatively. Bladder antisepsia was done daily on the catheter using 2 ml of methylene blue. Postoperative urinary output was monitored every 6 hours.

Postoperative analgesia was provided with one dose of butorphanol (Richterpharma AG Austria) (0.4 mg/kg SQ) administered with 15 minutes before recovery. Synulox - Pfizer Animal Health Italy (amoxyciline + clavulanic acid) in a dose of 8.75 mg/kg q 24 h, was administered for 10 days. Carprofen – Rymadil, Pfizer Animal Health Italy (4 mg/kg IV) was administered perioperatively.
Postoperatory at 8 days was observed urine evacuation besides urinary catheter. For 45 days after surgery the animal showed signs of urinary incontinence without any signs of ascending urinary infections. At recheck performed at 90 days the owner tells that the animal adopt the miction position urinating in continuous flow. There were no involuntary loss of urine.

Shortening of the neourethra predispose for bladder displacement caudally into the pelvic canal, fact found in male dog with urethral sphincter mechanism incompetence (17). In our bitch, postoperative positive-contrast studies demonstrated that the bladders were not displaced in the pelvis.

The animal shows a favourable evolution after surgical intervention at time (six months) when this paper was submitted for publication.

Discussions

Urinary incontinence is the main postoperative complication seen in vagino-urethroplasties performed after resection of urethral neoplasia and granulomatous urethritis (23). This complication has been reported in carnivores by other authors after traumatic ruptures of the urethra (1, 17). At female patients urinary incontinence was reported in 41 of 49 cases submitted to vaginal flap reconstruction of the urethra and bladder neck (Blaivas-1996), a procedure similar to that which was utilized in this case.

Relatively recent studies show that after urethrotomy pudendal nerve stimulation in distal urethra is unaffected and therefore, the continuity of bladder with urethra is not required for normal urethral closure mechanism (3, 12, 18). The remaining length of the urethra has a significant influence on postoperative continence and preservation of the dorsal vascular and nervous pedicles of the urethra is absolutely necessary to prevent urinary incontinence (20). In the conditions found in our case (the absence of urethra and an evolution from urinary incontinence to controlled miction in flow with low pressure) is likely that neck’s bladder urethral sphincter has not been affected.

Other palliative treatment procedures - reimplantation of the ureters in the proximal aspect of the vagina produce permanent urinary incontinence (10,19). Some owners accept this complication with the potential for local control (10).

Vagino-urethroplasty is a surgical procedure used preponderant after bladder and urethra cancer resections (23). In this cases the frequency of serious complications, including urinary incontinence and ascending lower urinary tract infections, was low because urethra is resected including the urethral tubercle, although extended to over 50% of the length of the urethra (White-1996), being not total as in the presented case.

Palliative treatment with a cystostomy tube can be used only as a short-term palliative measure (4, 21).

Procedures of urinary diversion by ureterocolonic, ureterojejunum or ureteroileal anastomosis produces frequent postoperative complications as: hyperchloremic acidosis, uremia, hyperammonemia, refluxing ureteric anastomosis, hydroureronephrosis, pyelonephritis (2, 14, 22).
Conclusions

In accordance to our knowledge, this report describes for the first time a urethral injury by iatrogenic surgical resection of the urethra in the bitch after vaginal hyperplasia surgery. Neourethral reconstruction by vaginal flaps procedure described should be considered as a palliative surgical alternative to urinary diversion or euthanasia. Postoperative urinary incontinence remains a potential problem after this procedure.

Acknowledgements

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References

The outstanding food properties of trout make it one of the most preferable fish around the world. Among the 40 species within the trout family, we decided to focus on three of the globally most common ones – rainbow, brown and brook trout. Presumably, the distinct origin of each species and different pathogen pressure in their natural habitat might have led to different levels of immune response. Complement system and its two major activation pathways – classical (CPCA) and alternative (APCA), plays huge role in organism’s defense. The goal of this project is to investigate the complement activity variations among these three trout species, reared under identical intensive technology. Although introduced in Europe not too far ago, the rainbow trout exhibited the second highest result for APCA activity and the highest one for the CPCA. These results explain farmers’ willingness to breed it in large quantities and correspond to the global domination of this trout type. Contrary the brown trout, which is widely distributed on the continent, presented the lowest possible results for both parameters of interest. Expectedly, the brook trout exhibited excellent results for both pathways. Despite considered very similar, the three fish species unveiled different immune response levels, which should lead to development of dedicated breeding strategies for each of them.

Key words: salmonids, trout, complement

Grown in fresh water ponds or raceways, the exceptional nutrient properties and great taste of trout makes it one of the most preferable and healthy meats on the market. Modern trout farming requires high density of the grown population and fast growing periods of the reared fish (4). This increases the risk of spontaneous outbreaks of infectious diseases with potential devastating effect. As opposed to the other divisions of agriculture, fish farming does not have enough options for disease control, which unveils the necessity of good immune defense (3). The main elements of innate immunity include the serum lysozyme, ß-lysinies and the complement system. For the purposes of this experiment, we decided to focus on the two major pathways of complement activation – classical and alternative.

The classical pathway of complement activation (CPCA) is part of the non-specific immune protection against variety of pathogens. The complement cascade is triggered via binding of antibodies to antigen, which makes CPCA extremely specific and irreplaceable part of the native immunity (5). In contrast, the alternative pathway of complement activation (APCA) kills bacteria via opsonization. The pathway is initiated by direct bind between the C3b protein and a microbe, which
makes it much faster than the classical pathway and one of the first protective mechanisms in case of pathogen occurrence (8). Even though CPCA is more accurate, it is suggested that APCA is of great importance of salmonids innate immunity (6). Alternative pathway of complement activation in salmonid fishes is capable of neutralizing enveloped viruses like infectious hematopoietic necrosis virus (IHNV) and the viral haemorrhagic septicaemia virus (VHSV), which endorse its crucial role in pathogen elimination (7).

The trout family includes more than 40 species, where the usual commercial production includes just a few of them. In this study, we decided to focus on three of the most common ones – rainbow, brown and brook trout. The three species belong to different trout genus, which gives them slightly different living cycle. (12). The ability to hybridize with other members of the trout family and strong immune system allowing it to out-compete them, gave the rainbow trout (*Oncorhynchus mykiss*) a reputation of invasive species (20). Brown trout (*Salmo trutta fario*) originates in Europe and is part of the Salmo genus. Unlike the rainbow trout, this salmonid fish spends its entire life in fresh-water rivers, hence it is considered purely fresh water trout - "river trout" (21). The brook trout (*Salvelinus fontinalis*) is a highly adaptive and variable species that is able to live in many different habitats.

Breed, sex and season fluctuations of innate immune response have been described in variety of species (14,15,16), which provoked us to elucidate the potential variations of the aforementioned two complement pathways within three species of trout.

### Materials and methods

The aforementioned three trout species - rainbow, brown and brook trout were grown under intensive technology in concrete tanks in private fish farm located near the town of Peshtera, Bulgaria. For the purposes of this project, 60 samples were obtained per each trout kind, where the total number of investigated fish was 180. Samples for the study were obtained from commercial size fish (>24 months old fish) within the month of November, when the water temperature was optimal for trout species (≈13.8°C).

Blood for analysis was obtained aseptically from the caudal vein (*v. caudalis*) using plain vacutainers. Prior sampling, fish were stunned electrically with mobile device consisting of a capacitor (condenser; low capacitance of 47 μF) and two copper plates (electrodes). Out of water, fish handling and blood samples were performed by wearing latex gloves to avoid skin damage.

The levels of the alternative pathway of complement activation (APCA) was assessed by the method of Sotirov (13). Each serum sample was first diluted by mixing 100μl serum with 350 μl veronal-veronal Na buffer (final concentrations: 146 mM NaCl, 1.8 mM 5.5-diethylbarbituric acid sodium salt; 3.2 mM 5.5-diethylbarbituric acid; 1 mM EGTA and 0.8 mM MgCl2), in U bottomed plates (Flow Laboratories, UK). From each diluted serum 7 serial dilutions were again prepared in veronal-veronal Na buffer: 80 μl diluted serum + 20 μl buffer, 70 μl diluted serum
+ 30 μl buffer, 60 μl diluted serum + 40 μl buffer, 50 μl diluted serum + 50 μl buffer, 40 μl diluted serum + 60 μl buffer, 30 μl diluted serum + 70 μl buffer and 20 μl diluted serum + 80 μl buffer. The final serum dilutions were, 8/45, 7/45, 6/45, 5/45, 4/45, 3/45 and 2/45, respectively. Then 50 μl buffer and 100 μl of 1% rabbit erythrocyte suspension were added to each well. After 1 hour incubation at 37°C the samples were centrifuged at 150 G for 3 minutes at room temperature (23°C). Thereafter, 150 μl of supernatant was removed from each well and placed in flat bottomed plates for measurement of optical density at 540 nm using “Sumal-PE2” ELISA reader (Karl Zeiss, Germany).

The classical pathway of complement activation (CPCA) was determined by the method of Stelzner and Stain (18). Each serum sample was first diluted by mixing 30 μl serum with 170 μl veronal-veronal Na buffer (in final concentrations: 146 mM NaCl; 1.8 mM 5,5-diethylbarbituric acid sodium salt; 3.2 mM 5,5-diethylbarbituric acid; 15 mM CaCl2 and 0.8 mM MgCl2) in U bottomed plates (Flow Laboratories, UK). From each diluted serum 5 other serial dilutions were again prepared in veronal-veronal Na buffer: 3/20, 3/80, 3/160, 3/320 and 3/640. Then, 100 μl buffer and 100 μl of 1% sheep erythrocyte suspension sensitized with haemolytic antibodies were added to each well. After 1-hour incubation at 37°C the samples were centrifuged at 150 G for 3 minutes at room temperature (23°C).

The last step for both pathways was to pipette out 150 μl of each supernatant in flat bottomed plates and measuring the optic density at 540 nm using a “Sumal-PE2” ELISA reader (Karl Zeiss, Germany). The final APCA and CPCA activity was calculated using a special computer software developed at Trakia University and expressed as CH50 units (CH50 units correspond to 50% of complement-induced haemolysis of applied erythrocytes).

The obtained data was statistically processed using the ANOVA single factor with confidence limits set at 95%, using the data analysis tool pack (Microsoft Excel 2016, Microsoft Corporation Ltd.).

Results and discussion

Phenotype diversity in the classical pathway of complement activation is presented on table 1. The highest CPCA activity was observed among the rainbow trout (92.51±1.98, CH50), while the lowest was detected for the brown trout population (67.06±4.04, CH50) (P<0.01). The brook trout exhibited the second highest result (89.13±3.15, CH50), which might be explained with the perfect adaptation of this species to our climate. The member of the Salmo genus had diametrically opposed levels of this pathway and exhibited significantly lowest result than the both trout (P<0.01), which motivates Bulgarian farmers to continue breeding this species.
Table 1

<table>
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<th>Species</th>
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<th>CPCA (CH50)</th>
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<td>60</td>
<td>92.51±1.98a</td>
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<td>Brown trout <em>(Salmo trutta fario)</em></td>
<td>60</td>
<td>67.06±4.04ab</td>
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<td>60</td>
<td>89.13±3.15b</td>
<td>12.05</td>
</tr>
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</table>

a,b – P<0.01

Although triggering mechanism for the alternative pathway of complement activation is quite different than the CPA, the results for the APCA activity were relatively similar (table 2). The highest results for this complement route were presented by the brook trout (701.55±2.19, CH50), followed by the rainbow trout (698.06±2.52, CH50). The close results between both species confirm the extreme importance of this indicator. Contrary, the brown trout displayed the lowest levels of APCA activity (628.10±3.77, CH50), where both the results of the rainbow and brook trout were significantly higher (P<0.01).

Table 2

<table>
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<td>698.06±2.52b</td>
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<td>628.10±3.77ab</td>
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<td>Brook trout <em>(Salvelinus fontinalis)</em></td>
<td>60</td>
<td>701.55±2.19a</td>
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</tbody>
</table>

a,b – P<0.01

The complement system is a key mechanism for organism’s defense against pathogens. It consists of plasma proteins that can be triggered directly or indirectly by pathogens and their associated antibodies. Therefore, immune capacity of fish can be demonstrated by high values of complement activity (6). Both alternative and classical pathways of complement activation within fish are comparable with the mammalian complement system. Interestingly, important components of the complement cascade, such as C3, are more polymorphic in fish than in mammals (19). Considering the important role of C3 component in the reaction’s cascade, the possible variations within this molecule could be the key to understand the variable results among the three investigated species from our project. Although being members of the trout family, each of them has different origin and conceivably slightly different genetic polymorphism regarding the complement system components.
Obtained data for both pathways of complement activation confirm previous results for rainbow and brook trout of other authors (9,17). Papezikova et al. (9) investigates the complement activity among brook trout, rainbow trout and the hybrid between them. Similarly, the brook trout exhibits the highest levels of complement activity, while the rainbow trout has significantly less CPCA activity. Interestingly the hybrid between them exhibits intermediate results. This confirms the polymorphism within the genes responsible for the complement cascade and their unpredictable inheritance (9).

Net confinement studies show significant modulation in APCA activity between rainbow and brown trout. Alternative complement activity and oxygen radical production were found almost twice as high in brown trout compared with the other trout fish. The diverse stress response and subsequent immunity variations are based on the different cortisol production within both species, which enhances or suppresses, respectively, the APCA activity (11).

Despite being introduced in Bulgaria just a few decades ago, the extremely high complement activity of the rainbow trout gives it better chances to survive and procreate itself in nature. These results somehow explain the total world domination of this trout kind and farmers’ readiness to increase the volumes of its production. The brook trout unveiled very high results for both traits of interest, which unambiguously shows its well-adapted immune system to the local environment. Though introduced in the second half of the previous century in Bulgarian aquaculture sector, this fish has extremely high capabilities for commercial production within high stocking density. Showing low values for both complement pathways, the brown trout has difficulties to compete with the other two trout in wildlife. A substantial increase in the brown trout’s immune defense might be achieved via the use of specific immune stimulant substances. Balcázar et al., (2) determined significantly higher results within brown trout treated with several lactic acid bacteria (LAB probiotics). Positive effect of probiotic administration (Lactococcus lactis CLFP 100 and Leuconostoc mesenteroides CLFP 196) to brown trout’s survival and immune performance was witnessed after experimental A. salmonicida infection (2). Currently, immune system stimulation is highly explored area, where diverse results are obtained (10). Therefore, positive effect of used substances should be well investigated.

Conclusions

The gained data acknowledges the high performance of the rainbow trout, the well adaptation of the brook trout to our climate and the moderate complement activity of the considered local brown trout. Even though considered similar, the three investigated species have their immune variances, which should be taken into account before rearing programs are established. The relatively low values for both parameters of interest should encourage farmers to use such immune enhancers to boost brown trout’s natural defense and consequently improve its performance traits.
Acknowledgment

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RELEVANCE OF COMPUTED TOMOGRAPHY EXAMINATION IN ELBOW DYSPLASIA IN DOG

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Summary
Elbow dysplasia (ED) is a common condition in large breed dogs, being one of the main causes of debilitating lameness and elbow osteoarthritis in those breeds. The pathology has a hereditary character and can involve the coronoid process, the anconeal process, the humeral condyle or can affect the elbow congruency. The gold standard for the ED diagnostics is the radiographic (Rx) examination, but having in consideration that the Rx examination require multiple exposure and do not offer a full image of the bone structure, we aimed to highlight the importance of computed tomography to identify the subtle changes that can appear in the elbow. The biological material was represented by 10 dogs with the age ranging from 10 months to 4 years in which the elbow radiography was not concluding. Before CT evaluation of the elbow, the dogs undergo a clinical evaluation and after that, they were tranquilized and put in sternal recumbency with the front legs straight. Taking in consideration that the CT is able to perform reconstruction of an image base on a given direction, this offer the possibility to have a better view of the intraarticular space and also to identify a possible osteophyte, which in Rx evaluation could be super or over imposed on other structures. The CT evaluation of the bone give the possibility to evaluate also the changes that appear in the bone cortex and the medullary part and can be a consequence of ED. The CT evaluation of the elbow is able to establish the finest degenerative process that can be missed by the radiographic exam.

Key words: dog, elbow, elbow dysplasia, Computed Tomography

Elbow dysplasia (ED) is a common condition, and also an increasing pathology, that is mainly found in large breed dogs. ED is a complex group of lesions including osteochondrosis, ununited anconeal process, fragmentation of the medial coronoid process, incongruency of the elbow joint structures, that will produce an early degeneration of the elbow (1). The most common findings are the fragmentation of the medial coronoid process (2, 3, 4, 5, 6, 7). The complication of ED is given by the progressive osteoarthritis (3, 4, 8, 9, 10, 11), which cannot be resolved surgically (8).

A study made in 2014 (7) demonstrate that the dogs that were initially noted by Orthopedic Foundation of Animals (OFA) with a grade 0 or 1 for ED where...
evaluated through CT scan and small lesions of osteochondrosis where visible on CT, the scan having a specificity of 75% compared with radiographic evaluation (1, 7).

The gold standard for the ED diagnostics is the radiographic (Rx) examination (9-17), but having in consideration that the Rx examination requires multiple exposure and do not offer a full image of the bone structure, we aimed to highlight the importance of Computed Tomography to identify subtle changes that can appear in the elbow.

Materials and methods

The biological material was represented by 10 dogs, with the age ranging from 10 months to 4 years in which the elbow radiography was not concluding. Before the imaging examination the dogs were clinical evaluated through gait inspection. The dogs present first degree lameness, the lesions being preponderantly located in one limb. For the radiographic and CT examination the dogs were sedated using Xylazine (Bayer, USA) 2% 4 mg / animal i.m., Midazolam (Bayer, USA) 5% i.v., Propofol (Pfizer, USA) 5 mg / animal by intravenous injection.

Image acquisition. The radiographic examination was performed using a TEMCO-GRX X-Ray device with Xmaru 1717SGC/SCC Flat Panel detector and Xmaru VetView acquisition software. The patients were positioned in latero-lateral decubitus and the Rx exposure was done latero-laterally with the elbow flexed at approximately 90 degree. The acquisition kilovoltage was set at 79 kV with 16 mAs. The DX digital acquisition modality had an image pixel spacing of 0.127/0.127 and fixed grid.

The CT examination was performed on a 16-slice helical CT scanner (Siemens Somatom Scope – Siemens Healthineers Global USA). The patients were positioned in ventral decubitus with the elbow flexed at 90 degree, on each side of the head, the scanning started from the middle of radius end ulna until the humeral joint, the scanner table moving cranio-caudally.

The images obtained during spiral acquisition have a 512x512 matrix, total collimation width of 2.4, spiral pitch factor of 1.2, using a bone reconstruction protocol with U90s convolution kernel. The parameters used for the elbow scan was 130 kV with 52 mAs, table speed of 2.0 and table feed per rotation of 2.9. Scanning time for elbow was of 98.64 s with a slice width of 1 mm. Multiplanar reconstruction was made using a bone reconstruction protocol at a 0.6-1 mm per slice.

The images were evaluate using the work station software and RadiAnt, a free software for DICOM image format reader.
Results and discussion

Even if the radiography is the golden standard in the diagnostic of ED, in case of subtle lesion like small fragments of the medial coronoid process, on the radiography the coronoid process will present a blurred cranial edge (15) and subchondral bone sclerosis that is not so evident on the radiography (17, 18, 19). Radiographic early sign in ED are degenerative and are not evident on the radiography, being dependent on the radiographic technique (3, 15). Degenerative osteoarthritis has a progressive aspect being dependent on the time when the radiography was made (9, 15). The most evident lesions on the radiography is the ununited anconeal process (UAP) and osteophyte prolipheration on the edge of the joint structures (fig. 1, 2, 3).

Fig. 1. Degenerative arthrosic process of the anconeal process

Fig. 2. Osteophitic prolipheration (white arrow) and elbow inconruency (black arrow)
Fig. 3. Ununited anconeal process (white arrow), periosteal reaction (black arrow), osteochondritis

Base on the radiographic examination the elbow is dysplasic if there are present arthritic changes, and the presence of one or more of the following: ununited anconeal process (UAP), osteochondritis dissecans (OCD), fragmentation of the medial coronoid process (FMCP), malformation or elbow incongruency (20).

The limitation of the radiological examination is given by the fact that the 2D images displayed on the radiography is in fact obtained from a 3-dimensional structure. That make a challenging evaluation of the mild elbow incongruency and identification of the osteochondral free bone displacement (20, 21, 22, 23). Studies show that radiography have a high sensitivity in detection elbow incongruency greater than 2 mm, regardless of the beam angle (20).

CT examination of the elbows in the dogs in which the radiography was not concluding show osteoarthritis degenerative changes in the elbow and OCD lesions. The CT have the capability to render the multiplanar reconstruction of the elbow removing the superimposing deficient found in radiography helping to a better examination of the tissue and bone architecture. The capability of adjusting the HU window level permit simultaneously evaluation of the bone and soft tissue (20) (fig. 4, 5, 6, 7). Virtual 3D rendering of the elbow is another efficient tool in case of surgery.
Fig. 4. OCD of the humerus (white arrow), degeneration and osteoarthritis (black arrow)

Fig. 5. UAP (black arrow), OCD (white arrow), osteoartitis (red arrow)

Fig. 6. Osteoartitis

Fig. 7. OCD and osteoarthritis
Conclusions

In our study, the CT examination of the elbow offer an extra information on the aspect and architectonics of the elbow element, helping in ED diagnostic. CT examination is able to identify the slightest degenerative changes in the bone structure and can establish an early diagnostic in case of ED. Many study show that CT could represent a gold standard in ED diagnostic (13-23), the limitation of the examination are given by the high purchase price for the equipment.

References

PRELIMINARY STUDIES CONCERNING THE OPTIMISATION OF POLYMERASE CHAIN REACTION TECHNIQUE FOR mtADN ANALYSIS OF APIS MELLIFERA

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Summary
Several molecular methods have been developed for Apis mellifera mtDNA studies. Although current data easily allow optimization of PCR, in some circumstances, the selection of the optimal kits of extraction and amplification involve the evaluation of them by using the specifically infrastructure and equipment’s available. Methodology of research consisted in PCR analyses of the honey bee’s mitochondrial DNA by using different extraction and amplification kits available on the Romanian market. Extraction had been done with three commercial kits: QIAamp Cador Pathogen Mini Kit, PureLink Genomic DNA Kits and MagNA Pure LC Total Nucleic Acid Isolation Kit. PCR amplification has been done with two commercial kits: Taq PCR Core and Platinum Pfx DNA Polymerase. The DNA extract was measured by using Qubit Fluorometric Quantitation (Qubit3.0 Fluorometer, Qubitds DNA HS Assay Kit). There were observed slightly higher values for the samples extracted with QiAamp Cador Pathogen Mini Kit. The same amplification thermal cycling program was used for both commercial amplification kits (one cycle of 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 55ºC for 1min., 72ºC for 2 min and 72ºC for 5min, and cooling cycle of 4ºC). As a conclusion, all commercial kits proved high quality standards and provided similar positive/negative results. However, in this study, the best electrophoretic bands were obtained with Taq PCR Core. Based on this results we are going to continue the research for Apis mellifera genetical analysis.

Key words: PCR, mtDNA, honeybee, Apis mellifera

Apis mellifera has a great natural distribution (14) and the variability of honey bee even in one colonies it has been observed from antiquity (12). Mitochondrial DNA is used as a molecular marker to study genetic diversity among Apis mellifera populations (1). Genetic techniques, including analysis of mitochondrial DNA (mtDNA) and microsatellite markers, offer reliable classification of honeybee subspecies.

All molecular methods for extracting Apis mellifera DNA should be optimized to provide the best results by using the specifically infrastructure and equipment’s available. Methods should be adapted to provide satisfactory results, with minimum cost and time but maintaining quality. Also the DNA extraction techniques should use low toxicity reagents (8).

The aim of this study was to find the best method for DNA extraction from Apis mellifera thorax, producing sufficient quantity of DNA without PCR inhibitors,
to allow PCR amplification and then sequencing (6) to test the biodiversity of *Apis mellifera* from different parts of Romania. Optimizing PCR reaction acts to improve some parameters, in order to enhance specificity and sensitivity (7).

**Materials and Methods**

Five honey bee samples (10 bees for each sample) were tested in order to optimise the protocol. All collected bees were stored at –80°C until used. The extraction of the DNA was realized from the whole thorax of each bee. We used Magna Lyser Green Beads and PBS to obtain the mechanical lyses of the thorax. Extraction had been done with three commercial kits: QIAamp Cador Pathogen Mini Kit, PureLink Genomic DNA Kits and MagNA Pure LC Total Nucleic Acid Isolation Kit with small changes made to the protocol kits considering the particularities of the samples. Protocol changes refers to the increase of incubation time with proteinase K and lyses buffer and also the incubation time of the elution buffer.

After that we measured the DNA extract by using Qubit Fluorometric Quantitation (Qubit 3.0 Fluorometer, Qubit dsDNA HS Assay Kit).

All commercial kits proved high quality standards and provided similar results but for DNA extraction in this study we decided to use QIAamp Cador Pathogen Mini Kit.

PCR amplification has been done with two commercial kits: Taq PCR Core and Platinum Pfx DNA Polymerase (6). The mtDNA COI-COII intragenic region was amplified in iCycler 3200 thermal cycler (BioRad, USA), using primers E2 (5’-GGCAGAATGCTGATTG-3’) and H2 (5’-CAATATCATTTGAGACC-3’) (1, 2, 3, 4, 6, 9, 10, 11, 13). For one reaction with Taq PCR Core kit were used 2.5 μl 10x Taq PCR Core Buffer (Qiagen, Germany), 1 μl dNTP 10 mM (Qiagen, Germany), 2μl MgCl₂ 25 mM (Qiagen, Germany), 0.5μl Taq DNA Polymerase (Qiagen, Germany), 0.5 μl primer E2 Forward, 0.5 μl primer H2 Reverse, 15μl RNase-free water. For one reaction with Platinum Pfx DNA Polymerase were used 2.5 μl 10X Pfx Amplification Buffer (Invitrogen, USA), 0.75 μl GeneAmp® dNTPs 10mM (Applied Biosystem, USA), 0.5μl Magnesium sulphate 50 mM (Invitrogen, USA), 0.25 μl Platinum™ Pfx DNA Polymerase (Invitrogen, USA), 0.5 μl primer E2 F, 0.5 μl primer H2 R and 16 μl RNase-free water. PCR was conducted by using 3μl DNA and 22 μl mix solution.

Primer annealing temperature setting was done by probing, after using an onlineTₘ calculator. By means of this computer is set within a temperature range of between 53°C and 57°C.

Thermal cycling program consisted: one cycle of 94°C for 3 min (initial denaturation), 35 cycles of 94°C for 1 min (denaturation), annealing primer temperature variable depending on the computer program for 1min, 72°C for 2 min (elongation) and 72°C for 5 min (final elongation) and cooling cycle of 4°C.
There were realised five PCR reactions for both commercial amplification kits and also for each commercial extraction kits starting from 53°C and continuing with 54°C, 55°C, 56°C and 57°C for primers annealing temperature.

**Results and discussion**

After extraction with the three commercial kits and measuring the DNA concentration with Qubit Fluorometric Quantitation (Qubit3.0 Fluorometer, Qubit dsDNA HS Assay Kit), there were observed slightly higher values for the samples extracted with QIAamp Cador Pathogen Mini Kit (table 1).

<table>
<thead>
<tr>
<th>DNA concentrations (ng/ml) from the honey bee extracts</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAamp Cador Pathogen Mini Kit</td>
<td>189</td>
<td>303</td>
<td>203</td>
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<td>158</td>
</tr>
<tr>
<td>PureLink Genomic DNA Kits</td>
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<td>263</td>
<td>184</td>
<td>140</td>
<td>158</td>
</tr>
<tr>
<td>MagNA Pure LC Total Nucleic Acid Isolation Kit</td>
<td>185</td>
<td>272</td>
<td>190</td>
<td>141</td>
<td>169</td>
</tr>
</tbody>
</table>

Even if we used extraction kit that have different principle for separation of nucleic acids (silica membrane and magnetic particles) the results regarding DNA concentration were satisfactory for all the extraction kits. Values were in the range of 140 ng/ml and 303 ng/ml. Because the values were slightly higher for the samples extracted with QIAamp Cador Pathogen Mini Kit, we decided to use this kit for subsequent extractions of our genetic studies.

The samples taken were amplified with Taq PCR Core kit and Platinum Pfx DNA Polymerase. An inadequate annealing temperature of the primer scan influence the quality of amplicons obtained. If the temperature is too high, can block the connections and primers annealing to the template DNA. If the temperature is too low, can result non-specific alignment of the primers on areas with low complementarity and thus the occurrence of artefacts (5).

After amplification we measured the concentrations of the amplicons and the results are listed in the tables 2-8.

<table>
<thead>
<tr>
<th>Amplicons concentration (ng/ml) obtained with Taq PCR Core kit amplification and QIAamp Cador Pathogen Mini Kit extraction according to annealing temperature primers</th>
<th>53°C</th>
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<th>55°C</th>
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<th>57°C</th>
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<td>Sample 2</td>
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<td>135</td>
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<tr>
<td>Sample 3</td>
<td>82</td>
<td>91</td>
<td>179</td>
<td>130</td>
<td>128</td>
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<tr>
<td>Sample 4</td>
<td>53</td>
<td>56</td>
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<td>89</td>
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<td>Sample 5</td>
<td>52</td>
<td>55</td>
<td>145</td>
<td>88</td>
<td>76</td>
</tr>
</tbody>
</table>
**Table 3**

Amplicons concentration (ng/ml) obtained with Taq PCR Core kit amplification and PureLink Genomic DNA Kits extraction according to annealing temperature primers

<table>
<thead>
<tr>
<th></th>
<th>53°C</th>
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<th>56°C</th>
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<tr>
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<td>124</td>
<td>134</td>
<td>169</td>
<td>153</td>
<td>149</td>
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<tr>
<td>Sample 2</td>
<td>88</td>
<td>108</td>
<td>208</td>
<td>127</td>
<td>131</td>
</tr>
<tr>
<td>Sample 3</td>
<td>75</td>
<td>83</td>
<td>171</td>
<td>123</td>
<td>120</td>
</tr>
<tr>
<td>Sample 4</td>
<td>50</td>
<td>52</td>
<td>132</td>
<td>81</td>
<td>69</td>
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<tr>
<td>Sample 5</td>
<td>51</td>
<td>50</td>
<td>138</td>
<td>80</td>
<td>70</td>
</tr>
</tbody>
</table>

**Table 4**

Amplicons concentration (ng/ml) obtained with Taq PCR Core kit amplification and MagNA Pure LC Total Nucleic Acid Isolation Kit according to annealing temperature primers

<table>
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<th></th>
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<td>171</td>
<td>155</td>
<td>150</td>
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<td>Sample 2</td>
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<td>Sample 3</td>
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<tr>
<td>Sample 5</td>
<td>52</td>
<td>50</td>
<td>140</td>
<td>82</td>
<td>71</td>
</tr>
</tbody>
</table>

**Table 5**

Amplicons concentration (ng/ml) obtained with Platinum Pfx DNA Polymerase amplification and QIAamp Cador Pathogen Mini Kit extraction according to annealing temperature primers

<table>
<thead>
<tr>
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<th>53°C</th>
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<tr>
<td>Sample 2</td>
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<td>Sample 4</td>
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<td>Sample 5</td>
<td>50</td>
<td>52</td>
<td>97</td>
<td>68</td>
<td>62</td>
</tr>
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</table>

**Table 6**

Amplicons concentration (ng/ml) obtained with Platinum Pfx DNA Polymerase amplification and PureLink Genomic DNA Kits extraction according to annealing temperature primers

<table>
<thead>
<tr>
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<th>55°C</th>
<th>56°C</th>
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<tbody>
<tr>
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<td>134</td>
<td>137</td>
<td>99</td>
<td>97</td>
</tr>
<tr>
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<td>105</td>
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<td>Sample 5</td>
<td>51</td>
<td>52</td>
<td>92</td>
<td>64</td>
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</table>
Table 7

Amplicons concentration (ng/ml) obtained with Platinum Pfx DNA Polymerase amplification and MagNA Pure LC Total Nucleic Acid Isolation Kit extraction according to annealing temperature primers

<table>
<thead>
<tr>
<th></th>
<th>53°C</th>
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</tr>
<tr>
<td>Sample 5</td>
<td>50</td>
<td>52</td>
<td>93</td>
<td>65</td>
<td>57</td>
</tr>
</tbody>
</table>

Table 8

Amplicons concentration (ng/ml) obtained with Taq PCR Core kit amplification and Platinum Pfx DNA Polymerase amplification and QIAamp Cador Pathogen Mini Kit extraction with the same amplification thermal cycling program was used for both commercial amplification kits (one cycle of 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and 72°C for 5 min, and cooling cycle of 4°C)

<table>
<thead>
<tr>
<th></th>
<th>Taq PCR Core kit</th>
<th>Platinum Pfx DNA Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>163</td>
<td>142</td>
</tr>
<tr>
<td>Sample 2</td>
<td>216</td>
<td>131</td>
</tr>
<tr>
<td>Sample 3</td>
<td>179</td>
<td>155</td>
</tr>
<tr>
<td>Sample 4</td>
<td>140</td>
<td>98</td>
</tr>
<tr>
<td>Sample 5</td>
<td>145</td>
<td>97</td>
</tr>
</tbody>
</table>

Values were in the range of 50 ng/ml and 216 ng/ml. At 55°C annealing temperature primers, there was obtained the best concentration of the amplicons with both amplification kit and for all three extraction kits, with slightly elevated values for the samples extracted with QIAamp Cador Pathogen Mini Kit.

Comparing the two amplification kits, there were noted slightly higher values with Taq PCR Core kit. Lower temperatures than 55°C and higher temperature than 55°C, provided smaller amplicon concentrations.

Agarose electrophoresis was performed to visualize the PCR products [0.9 g agarose, 60 ml TAE/TBE 1x, 5 μl ethidium bromide; 10 mg/ml; 100V; 1.5A (7), 50 minutes, using 8 μL of amplicon and 3μl of sample loading buffer (5X) (BioRad, SUA), and EZ Load 100 pb PCR Molecular Ruler (BioRad, USA). All gels were visualised under UV on a Gel Doc (BioRad), using the Quantity One programme.
Fig. 1. Electrophoresis samples amplified with Taq PCR Core kit and extraction with QIAamp Cador Pathogen Mini Kit.

Fig. 2. Electrophoresis samples amplified with Taq PCR Core kit and extraction with QIAamp Cador Pathogen Mini Kit.

Fig. 3. Electrophoresis samples amplified with Platinum Pfx DNA Polymerase and extraction with QIAamp Cador Pathogen Mini Kit.

Fig. 4. Electrophoresis samples amplified with Platinum Pfx DNA Polymerase and extraction with QIAamp Cador Pathogen Mini Kit.
The migration was at 536 pb, in accordance with the previous studies (2). The intensity of the bands were correlated with the amplicons concentration. The most intense bands were obtained on 55°C temperature annealing primers for both amplification kits, but slightly more intense at Taq PCR Core kit. For temperature smaller than 55°C the bands intensity were lower than the bands intensity obtained with temperature higher than 55°C.

Conclusions

All commercial kits proved high quality standards and provided similar positive/negative results. However, in this study, the best electrophoretic bands were obtained with Taq PCR Core and with the following amplification thermal cycling program: one cycle of 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min., 72°C for 2 min and final extension one cycle 72°C for 5min, and cooling cycle of 4°C.

Based on this results we are going to continue the research for *Apis mellifera* genetically analysis.

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EVALUATION OF SERUM VALUES OF TOTAL PROTEIN (PT), ALBUMIN, TOTAL GLOBULIN AND IMMUNOGLOBULIN (IG) G, A, M, AND E IN CANINE DEMODICOSIS

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Summary
Demodicosis remains one of the main disease which affects the dog skin. Although in recent years there have been significant advances in the understanding of this important ectoparasitosis, more research in areas, such as pathogenesis and immunology is clearly needed. In this context, the aim of the present study is to determine the serum values of total proteins (PT), albumin, total globulin and immunoglobulins (Ig) G, A, M and E in the three clinical evolution forms of canine demodicosis. 67 dogs diagnosed with demodicosis were selected for study. The turbidimetric and colorimetric techniques revealed changes of PT and Ig serum values in dogs with demodicosis, as well as differences in serum Ig and PT concentrations between the three groups under study (LD, GD and PD).

Key words: canine demodicosis, total protein, albumin, total globulin, immunoglobulin

One of the leading factors in the evolution of canine demodicosis is the disorders of the immunological mechanisms (1, 4, 6). Studies on cellular immunity underline the existence of a T lymphocyte deficiency (3). The humoral immunoassay studies demonstrate that in serum of dogs with demodicosis, PT and Ig values are altered whereas in clinically healthy dogs these values are included in the reference limits (5).

There are authors (6, 7) who support the existence of a vicious circle in which the disturbance of the humoral factor negatively influences the activity of T lymphocytes which will allow multiplication of the mite due to their inability to specifically recognize Demodex canis. The mite will lead by multiplying it to disorders of the cell-mediated immune system, as well as changes in humoral components.

Recent studies (2) emphasize the involvement of cholinergic pathways in association with cytokines regulation as an insight into the immuno-pathogenesis of canine demodicosis. Findings of the present study evidently suggest that Demodex mites might be affecting the cholinergic pathways to induce immunosuppression in their host and then proliferate incessantly in skin microenvironment to cause demodicosis (2).
Starting from this bibliographic information, the aim of the present study is to determine the serum values of total proteins (PT), albumin, total globulin and immunoglobulins (Ig) G, A, M and E in the three clinical evolution forms of canine demodicosis.

Materials and methods

67 dogs diagnosed with demodicosis were selected, their anamnesis and epidemiological data suspected the presence of immunosuppressive factors. The dogs studied were divided into three groups: group 1 consisting of 25 dogs with localized demodicosis (LD); group 2 of 20 dogs with generalized demodicosis (GD); group 3 consisting of 22 dogs with piodemodicosis (PD).

To these lots is added group 4 (the control group) of 20 clinically healthy dogs.

Blood samples were collected to determine serum IgG, IgA, IgM and IgE values using turbidimetric technique. Serum levels of total proteins, albumin and globulin were obtained by the colorimetric method.

Results and discussions

The turbidimetric and colorimetric techniques revealed changes of PT and Ig serum values in dogs with demodicosis, as well as differences in serum Ig and PT concentrations between the three groups under study (LD, GD and PD).

**Group 1**, 25 dogs with localized demodicosis, shows an increased albumin concentration accompanied by a decrease in the total globulin to 72% of the 18 individuals. This situation has led to an increase in the total protein concentration (the ratio of elevated albumin and low globulin). In seven dogs (28%) serum PT, albumin and globulin serum values were within the reference values. In correlation with the decrease in total globulins, IgG (15 dogs - 60%) and IgA (in 17 individuals - 68%) decrease significantly. The decrease in these Ig is accompanied by an increase in IgE concentration in 76% of all dogs (19 individuals). IgM did not show any changes in the values compared to the control group, falling within the reference limits for all individuals in group 1 (25 individuals - 100%). 16% of individuals in group 1 did not show changes in immunoglobulin and total protein values compared to the control group (Table 1).

In **group 2** (20 dogs with generalized demodicosis), the albumin levels show elevated values in 12 individuals - 60%, while the total globulin serum values fall below the baseline to 14 individuals in the group (70%). The albumin / globulin ratio leads, in this situation, to a PT increase to 55% of the batch (11 individuals). Six dogs (30%) show normal values of all parameters studied. IgE growth is also noted in the group diagnosed with DG; In 12 individuals in this group (60%) this increase is observed. IgM has normal values in 18 individuals in this group (90%). A "clean" immunogram is found in five individuals (25%) of group II (Table 1).
The results obtained in group 3 of 22 dogs diagnosed with piodemodicosis revealed differences compared to the situation of groups 1, 2 and the control group: to decrease albumin in 12 individuals (55%), accompanied by an increase in total globulins in the same individuals (55%). These values determined a decrease in total protein to 36% of the total group. Normal values of the three parameters studied (albumin, total globulin and total protein) were found to be 36% of the whole group.

IgG shows high values in 14 dogs of the group (64%), and IgA and IgE record increased values in 13 individuals (59%). IgM is maintained at normal levels in 91% of individuals in group III. Only four dogs out of 22 had unchanged IgG, IgM, IgA and IgE values compared to the control group (Table 1).

Table 1

<table>
<thead>
<tr>
<th>No</th>
<th>Evolution form</th>
<th>Cases</th>
<th>PT (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>Globulin (g/dl)</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LD</td>
<td>25</td>
<td>7.46</td>
<td>4.46</td>
<td>3.0</td>
<td>552.08</td>
<td>16.6</td>
<td>176.84</td>
<td>0.00064</td>
</tr>
<tr>
<td>2</td>
<td>GD</td>
<td>20</td>
<td>7.46</td>
<td>4.55</td>
<td>2.91</td>
<td>482.85</td>
<td>16.3</td>
<td>208</td>
<td>0.00059</td>
</tr>
<tr>
<td>3</td>
<td>PD</td>
<td>22</td>
<td>5.84</td>
<td>2.0</td>
<td>3.83</td>
<td>840.18</td>
<td>43.13</td>
<td>215.18</td>
<td>0.00057</td>
</tr>
</tbody>
</table>

The results of this study reveal increased concentrations of total proteins in the serum of dogs diagnosed with dry demodicosis (64%) (Table 1).

In dogs with piodemodicosis, PT values decrease (36%). The increase in total protein in localized and generalized demodicosis is explained by an increase in albumin over total globulins which remain at low concentrations in both clinical forms of disease. Small differences are noted between the total globulin values in localized demodicosis (72%) and those in the serum of dogs diagnosed with generalized demodicosis. The apparent decrease of total proteins in piodemodicosis is explained by the decrease of albumin and the significant increase of total globulins due to bacterial superinfections.

For some authors (1, 4, 7), the increase in serum proteins evokes an immediate hypersensitivity phenomenon corresponding to the increase in the percentage of circulating antibodies.

Determination of serum β-globulin (IgG, IgA, IgM and IgE) revealed a decrease in PT in DL and DG, associated with a decrease in IgG and IgA concentrations. In PD, IgG records increased values compared to the control group (64% of the group). The increase in serum concentrations in PD is also noted for IgA. Significant differences in serum IgG and IgA are noted between dry demodicosis dogs (groups 1 and 2) and dogs with piodemodicosis (group 3). The apparent decrease of IgG and IgA in groups 1 and 2 and the increase in the concentration of these immunoglobulins in group 3 are correlated with PT variations recorded in the three groups under study. In all clinical forms, IgE records increased values compared to the control group (from 0.0006 to 0.008 mg/
There were no significant differences in IgE concentrations between the three groups of dogs. IgM values correspond to the reference values and did not vary significantly between the three groups.

Canadian author studies (6) do not agree the implication of humoral factor (Ig) in the multiplication of the mite and the onset of demodicosis. They support the existence of a cellular immune deficiency (T lymphocytes) responsible for the multiplication of the mites, and the decrease in serum Ig levels does not predispose dogs to demodicosis and can not be accepted as an important cause of the disease.

Increases in serum Ig and total globulin concentrations in PD lead to a marked decrease in PT in this form of clinical evolution. Responsible for this process are the pyodermic bacteria associated with Demodex mite, which will cause a cellular immune deficiency during disease progression (4).

By comparing the results of the present study with that of other researchers, we can affirm that, the canine demodicosis is a complex phenomenon involving the intervention of several factors. Decreased serum Ig does not cause demodicosis; make the disease and those dogs whose Ig falls within the reference values. When the decrease in serum immunoglobulins is determined by the action of various factors (maintenance, nutrition, intercurrent illness) and/or immunodepression (weaning stress, estrogen, parturition, surgical interventions, hypercorticotherapy) or when the decrease in serum Ig is accompanied by a mediated immune deficiency (T lymphocyte deficiency) in the presence of permanent antigenic stimulation (Demodex canis), then the occurrence of the localized disease is imminent.

Extending this situation leads to generalization of the disease and to maintaining Ig at low values compared to Ig values in healthy dogs. When bacterial infections overlap and the disease acquires a piogenic character, Ig serum values are reversed by the intervention of the new immunosuppressive factors; In this situation, the increase in serum immunoglobulin levels is maintained by the parasite itself. The steady increase in IgE in all three forms of clinical evolution may evoke the involvement of IgE in a hypersensitivity phenomenon overlapping with a humoral or cellular immune deficiency.

Conclusions

In dry demodicosis (LD and GD), serum levels of albumin (66.66%) and total protein (64.44%) increase and total globulin values decrease (71.11%). Dogs with PD have low total protein concentrations (36.36%) and albumin (54.54%), accompanied by total globulin growth (54.54%).

No significant differences in serum concentrations of total proteins, albumin and total globulin between group 1 (LD) and 2 (GD) are noted.

Serum concentrations of IgG and IgA are low in LD and GD, with small differences in these parameters between the two groups and increase IgG (63.63%) and IgA (59.09%) in piodemodicosis. Serum concentrations of IgE
increase steadily in canine demodicosis in all three forms of clinical evolution (groups 1, 2 and 3); 94% of the dogs studied had IgM concentrations within the reference range.

Acknowledgements

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References

CELLULAR CYCLE CONTROLled BY INTRACELULAR LEVELS OF cAMP IN MAMMALS

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Summary
The key molecular in meiosis control is the 3′-5′-cyclic adenosine monophosphate (cAMP), a single-phosphate nucleotide involved in signaling intracellular transduction. There are many researches aiming the evaluation of cAMP importance in oocytes and cumulus-oocytes complex, with or without granulosa cell participation. Gonadotropins and 17β-estradiol stimulate the production of cAMP in follicular cells. Cyclic AMP is produced in cumulus and granulosa cells, as a consequence of ATP dephosphorylation under the action of adenylate cyclase – enzyme present also in oocyte. The intracellular signaling is depending on first and second messengers, which are different as chemical structure, and passing the membrane cell differently due to its solubility towards the bilayer membrane structure. The second messenger is very important because the first messenger cannot cross the phospholipid bilayer of the cell, because of its physical characteristics, being hydrophilic molecules. Thus, the limitative functionality of the signaling needs the second messenger which can cross the bilayer membrane from extracellular to intracellular compartment. Cyclic AMP dependent pathways participate actively to regulation of steroidogenesis in gonadal and adrenal cells, being considered as the second intracellular messenger in proliferation and differentiation and also in different physiological situations (migration, survival, apoptosis).

Key words: cAMP, cellular cycle, mammals.

The accurate control of cell cycle for development and growth is decisive for the final size and shape of tissues in multicellular organisms. Cell replication involves an elaborate pathways network of signaling compounds which processes extracellular messages regarding the identity and characteristics of the neighbor cells. These informations guide the development program which manages the cell size and function (10, 13, 14, 15).

Meiosis cell division is the specific mechanism which allows the formation of fecundable gametes as a result of reductional division of the genetic material. In the case of the female, the egg contains half of the chromosomes specific for every animal species, with n number of chromosomes. In bovine, while the time the oocyte is located inside the follicle, the oocyte remains stuck in the diakinesis stage.
of the first meiotic division (prophase, nucleus 4n). At this stage the chromosomes are present in a double copy as diffuse form (19), remaining in this phase until ovulation or degeneration of the oocyte occurs (6, 17).

The oocyte will have the ability to begin and complete the graduated form of meiosis during folliculogenesis (17). In vivo, preloading of LH induces the necessary follicular changes that allow the oocyte to resume the process of nuclear maturation.

Oocytes that have completed their growth are capable of restarting the meiotic division of the spontaneous form, when they are separated from inside of the correspondent follicle. This situation is observed in all studied mammalian species and occurs also in the absence of gonadotropins (13, 17). This information supports the hypothesis that the follicle produces one or more factors that prevent restoration of meiosis. Some experiments in this direction have demonstrated that follicle inhibiting action is localized in granulosa cells (20). In addition, it has been shown that the inhibitory potential is manifested by the clear form when it is possible to restore the connection between the oocyte-cumulus complex with the granulosa cells (16, 21).

One of the controlling mechanisms involves the presence and the action of cAMP. Thus, the cAMP is involved in various direct or indirect metabolic pathways. In meiosis control, the cAMP is a key molecule (7, 3). Several experiments have performed to evaluate the importance of oocyte cAMP and oocyte-cumulus cell complex with or without granulosa cells. Gonadotropins and 17-β-estradiol stimulate the production of cAMP in follicular cells (17).

Thereby, cAMP is produced in cumulus and granulosa cells as a consequence of ATP dephosphorylation by adenylate cyclase [8] which is the enzyme also present in oocytes (9). Cyclic adenosine monophosphate acts as a second messenger in steroidogenesis (17) and involved in the function of inhibitor meiosis factors produced by granulosa cell, such as: “oocyte maturation inhibitor” and the maturation inhibitor factor (MIF) (7, 24).

Some researchers have demonstrated by experiments on rats that a significant decrease in the number of union between cumulus and granulosa cells. This process is induced by LH reovulat discharge or by the addition of gonadotropins to the culture medium responsible for the cessation of the meiotic blockage exercised by the follicle (17, 25). Contrary to other species, meiosis reinitiation seems to depend on protein synthesis and the production of specific factors (17, 23).

Some in vitro experiments demonstrate that intraoocyte cAMP concentration can be modulated by the addition of various factors from the culture medium. Thus, purines and other inhibitors of phosphodiesterase (enzyme responsible for cAMP degradation) are frequently used to avoid spontaneous resuming of nuclear maturation, but with different efficacy.

Hypoxanthin – the substance present in bovine follicular fluid (8) is poorly effective for blocking meiosis in bovine oocytes, contrary with what happens in mouse oocytes (5, 19). But IBMX (isobutyrylthioguanosine) it is very effective, although its action is short because it is the only way to get the re-establishment of the cell division (22).
Another form of modulation of cAMP production is achieved through intracellular Ca\(^{2+}\) levels. Thus, the releasing of deposited calcium in the endoplasmic reticulum activates calmodulin, which in turn stimulates phosphodiesterase activity. By preventing calcium from activating calmodulin (or inhibiting calcium release via the inositol phosphate pathway, there is a decrease in cAMP degradation rate preventing the decrease of its intracellular concentration (7).

The cellular cytoplasm contains various protein kinases, from which protein kinase A is cAMP-dependent. The regulatory portion of this protein kinase is withdrawn from the molecule by cAMP, as a form that activates the catalytic subunit. Few prior to germinal vesicle rupture, a decline of the protein kinase A (PKA) activity is registered associated with a decrease in cAMP concentration (10, 12, 22). It is possible that the decrease of protein kinase A activity to allow the activation of a maturation factor (maturation promoter factor – MPF) (2).

In other species, namely *Xenopus* frogs, it was demonstrated that there is a protein kinase C (PKC) independent of cAMP, which is also involved in the control of meiosis and, implicitly, of the cell cycle mediation (24).

In *vitro* bovine oocyte can be mediated by protein kinase A and protein kinase C which can be activated by Sp-cAMP or phorbol myristate acetate, or inhibited by Rp-cAMP, sphingosine or forkolin. A short time treatment to activate PKC mimics the r-hFSH effect on cytoplasmic maturation (1).

### Materials and methods

The experiment was conducted on bovine oocytes cultured in the maturation medium from 14 to 24 hours, which consisted of the control group and the experimental groups. In our experimental study we analyzed the nuclear stage of incubated oocytes at 14, 16, and 18 hours in the maturation environment, followed by treatment with cell cycle protein synthesis inhibitor for 12 hours, and a final incubation in maturation medium supplemented with 3 mMol cAMP associated with IBMX 1 mMol.

At the end of each maturation period, they were denuded and then fixed and examined with a microscope. The results were statistically analyzed with the X\(^2\) test (4).

### Results and discussions

The results obtained in our experiment indicate that the effect of oocyte treatment with cAMP 3mMol and IBMX 1mMol depends on the time when the protein synthesis inhibitor is introduced. Thus, the effect is very small when induced early (at 14-16 hours of maturation) and is very high when induced at 16-18 hours of maturation. There are significant differences between maturation hours (X\(^2\), p <0.05).

Simultaneous addition of cAMP and IBMX to preincubated oocytes for 18 hours and posterior treated with protein synthesis inhibitor maintains the oocytes in...
interphase. So, the result is a high rate of interphase oocytes (82%), fact maintained even when the protein synthesis is restored. This demonstrates that together cAMP and IBMX are able to keep the bovine oocytes in the interphase. The inhibitory effect of the cell cycle protein synthesis inhibitor and the associated effect of cAMP with IBMX on meiotic maturation are reversible, even if this reversibility is conditioned by the duration of the exposure period and the nature of the additional components.

In rats, variation in cAMP concentration is mainly regulated by phosphodiesterase inhibitors. Our experimental results indicate that cAMP prevents chromosome condensation in bovine oocytes once it has been re-initiated. This demonstrates the ability of cAMP to maintain bovine oocytes in the interface, their effect being more pronounced when maturation is done later. Protein kinase A plays a very important role in meiotic arrest — prophase I and thus preventing progression in metaphase II (figure 1), allowing the development competence of oocyte for fertilization (11).

![Cellular cycle control and cAMP](image)

This could be explained by the inhibitory effect of cAMP on MPF factors (responsible for chromosome condensation once protein synthesis has been re-initiated). For all of this, phosphorylation of specific proteins may play a strong role in the transition from interphase to metaphase II. This phosphorylation is inhibited by the action of cAMP and IBMX.

**Conclusions**

Meiosis cell division is controlled and mediated by various components, and one of the key substances is cAMP. Thus, cAMP is presented in cumulus and granulosa cells, due to the dephosphorylation of adenosine triphosphate (ATP), mediated by the specific enzyme from oocytes (such as: adenylate cyclase).
The chromosomes of oocytes treated with protein synthesis inhibitor remain decondensed when added to cAMP and IBMX culture medium, and decondensation is maintained even if protein synthesis has resumed. These results demonstrate that the association between cAMP and IBMX inhibits oocyte development, conditions necessary for the transition to metaphase. These observations can be applied to solve the problems resulting from the desynchronization of the meiotic cell cycle.

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References


DOLPHIN WELFARE ASSESSMENT IN CAPTIVITY BASED ON BLOOD BIOCHEMICAL INDICATORS

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Summary
Keeping dolphins in captivity health and well-being requires an assessment permanent full health through clinical and laboratory. Study was done on two female dolphins aged around seven years, living in the same environment. From the two females blood samples were taken which were determined in the blood twelve biochemical parameters: creatinkinase, alkaline phosphatase, creatinine, magnesium, total serum protein, serum iron, lactic acid, serum amylase, cholesterol, glucose, lipase and serum triglycerides. Blood biochemical parameters were determined by dry biochemistry techniques, using the Vettest8008. The obtaining results reveal a decrease in the values determined: creatinkinase, alkaline phosphatase, total serum protein, sideremia, serum glucose which suggests a slight stress of captivity and environmental well-being.

Key words: dolphins, welfare, biochemical parameters, blood samples

The well-being of dolphins in captivity is very important and can be full when their living and feeding environment is correlated with physiological and behavioral needs, avoiding stress, illnesses and negative psychological states (1, 6). Dolphins in captivity must be provided with adequate space, adequate water quality, optimal microclimate parameters (light, temperature, ventilation, noise), appropriate feed quality and quality. To avoid health problems, the water in which dolphins live must have a NaCl concentration of 25-35 ‰, with a pH of 7.5-8.2, be clear and devoid of toxic chemicals (4, 5). The comfort temperature is 10 to 28 °C, the photoperiod is 1: 1, the ventilation provides a relatively uniform distribution of clean air and avoids strong currents, to avoid high intensity noise to minimize stress (3, 6).

Swimming pools must be non-abrasive, easy to clean, resistant to physical and chemical agents. Feeding dolphins from captivity is done with herring, mackerel and chapel.

The assessment of captive dolphin welfare over time has been made on the basis of the quality of life, diet and behavior. In this study, the study aims at assessing well-being based on the biochemical parameters determined (1).
Materials and methods

The research was carried out within the Constanta Museum of Natural Science Museum, on two elderly females (about 8 years old) (Nei-Nei and Cheng-Cheng). The Nei-Nei woman has a body length of 2.55 m and a weight of 240 kg, and Cheng-Cheng has the same length and weight of 270 kg. The difference between the two lies in the length of the shorter rook at Cheng-Cheng and the dominant attitude of this female in relation to Nei-Nei. The pool where the two females are housed is 22 m long, 10 m wide and 3 m deep. The water temperature is 20 °C, the relative air humidity is 76 - 78 %. Dolphin food consists of the daily consumption of 7 - 8 kg of fish.

To determine blood biochemical parameters from the two dolphins blood samples were taken from the caudal vein. The following biochemical parameters were determined from the samples obtained by dry biochemistry using Vettest 8008: creatinkinase, alkaline phosphatase, creatinine, total serum protein, sideremia, lactic acid, serum amylase, cholesterol, serum glucose, lipase, magnesium, Potassium, sodium, phosphorus, calcium, serum albumin, total bilirubin and alanine aminotransferase. The results obtained were compared with the reference values in the literature.

Results and discussions

The obtained results of the 19 blood biochemical parameters determined from the blood samples taken from the two captive dolphin females can be found in Table no. 1. Analyzing the results obtained there are small differences between the two females accommodated and fed under the same conditions.

The 8-year-old Cheng-Cheng female with predominant behavior had exceeded the serum magnesium concentration approximately 1.5-fold, sodium 1.06-fold and total bilirubin-approximately 2.63-fold. Also, some blood biochemical parameters recorded values below the base line: creatine kinase 2.8-fold, alkaline phosphatase 1.04-fold, 1.07-fold serum glucose and 3-fold alanine aminotransferase.

The Nei-Nei female, aged approximately 7 years, had different outcomes compared to the baseline, with a less than 1.01 fold lower and alanine aminotransferase less than 1.17 fold. Higher than baseline values were observed for serum amylase approximately 2-fold, for sodium about 1.06 times, for serum albumin 1.08 times, and for total bilirubin 1.7-fold. Nei-Nei female values were also recorded at the upper reference limit for magnesium and serum calcium.

The other determined biochemical parameters were recorded in the reference values for both females. Variation in blood biochemical parameters indicates the associative existence of a form of renal and hepatic impairment without major alteration of specific functional parameters as well as the presence of captive stress.
Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Determined parameter</th>
<th>Results Cheng-Cheng</th>
<th>Results Nei-Nei</th>
<th>UM value</th>
<th>References value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Creatinkinase</td>
<td>89</td>
<td>114</td>
<td>UI/L</td>
<td>100 - 250</td>
</tr>
<tr>
<td>2.</td>
<td>Alcaline phosphatase</td>
<td>287,35</td>
<td>518,02</td>
<td>UI/L</td>
<td>300 - 1300</td>
</tr>
<tr>
<td>3.</td>
<td>Creatinine</td>
<td>1,86</td>
<td>1,4</td>
<td>mg/dl</td>
<td>1 – 2</td>
</tr>
<tr>
<td>4.</td>
<td>Total serum protein</td>
<td>6,4</td>
<td>7,4</td>
<td>g/dl</td>
<td>6 – 7,8</td>
</tr>
<tr>
<td>5.</td>
<td>Sideremia</td>
<td>145,08</td>
<td>118,7</td>
<td>µg/dl</td>
<td>120 - 340</td>
</tr>
<tr>
<td>6.</td>
<td>Lactic Acid</td>
<td>3,46</td>
<td>2,46</td>
<td>mmol/l</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Serum Amylase</td>
<td>201,0</td>
<td>211,0</td>
<td>mg/dl</td>
<td>150 – 260</td>
</tr>
<tr>
<td>8.</td>
<td>Serum Glucose</td>
<td>84,05</td>
<td>117,09</td>
<td>mg/dl</td>
<td>90 – 170</td>
</tr>
<tr>
<td>9.</td>
<td>Lipase</td>
<td>11,72</td>
<td>6,92</td>
<td>UI/L</td>
<td>1 – 16</td>
</tr>
<tr>
<td>10.</td>
<td>Magnesium</td>
<td>2,1</td>
<td>1,82</td>
<td>mg/dl</td>
<td>1.1 - 1.8</td>
</tr>
<tr>
<td>11.</td>
<td>Chlorine</td>
<td>118,21</td>
<td>118,29</td>
<td>mmol/l</td>
<td>113 – 125</td>
</tr>
<tr>
<td>12.</td>
<td>Potassium</td>
<td>3,64</td>
<td>3,53</td>
<td>mmol/l</td>
<td>3,2 – 4,2</td>
</tr>
<tr>
<td>13.</td>
<td>Sodium</td>
<td>163,4</td>
<td>162,59</td>
<td>mmol/l</td>
<td>153 – 158</td>
</tr>
<tr>
<td>14.</td>
<td>Serum phosphate</td>
<td>4,71</td>
<td>3,84</td>
<td>mg/dl</td>
<td>4 – 6</td>
</tr>
<tr>
<td>15.</td>
<td>Alanine aminotransferase</td>
<td>9,0</td>
<td>24,0</td>
<td>UI/L</td>
<td>28 – 60</td>
</tr>
<tr>
<td>16.</td>
<td>Serum Albumin</td>
<td>4,86</td>
<td>5,7</td>
<td>g/dl</td>
<td>4,3 – 5,3</td>
</tr>
<tr>
<td>17.</td>
<td>Total Bilirubin</td>
<td>0,35</td>
<td>0,34</td>
<td>mg/dl</td>
<td>0,1 – 0,2</td>
</tr>
<tr>
<td>18.</td>
<td>Serum Calcium</td>
<td>9,7</td>
<td>10,33</td>
<td>mg/dl</td>
<td>8,5 – 10,0</td>
</tr>
</tbody>
</table>

The variation in the biochemical parameters determined for the two captive dolphins is, from this point of view, well-wise as medium.

Conclusions

Hepatobiliary markers (alkaline phosphatase and alanine aminotransferase) indicate the associated existence of a form of hepatic failure without major alteration of specific functional parameters.

Blood chemistries: magnesium, total serum protein, serum amylase, sodium, albumin, bilirubin, serum calcium slightly exceed the reference values indicating a possible renal failure.

Low values of blood biochemical parameters: creatine kinase, alkaline phosphatase, sideremia, serum glucose may suggest the presence of captive stress.

Changes in blood biochemical parameters relative to baseline values indicate medium welfare of captive dolphins.
References


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Summary

For the moment, we are witnessing continued growth of consumer demands and requirements regarding the degree of freshness and sanitation of dairy products, which require major revisions of the methodology and equipment used for testing and analysis of commodity milk. In this study we intend the evaluation of some rapid test methods in the processing unit, the hygienic-sanitary parameters of the commodity milk regarding the establishment of the correlation SCC-CFU (somatic cell count, colonies forming units) for monitoring the health of commodity milk. According to the proposed objectives, this study is focused on assessing the relevance of automatic type systems Ekoskope and Soleris in cytological evaluation (SCC) and microbiological (CFU) on commodity milk processed into a company from Valea Gurghiului. The tests were conducted on samples of raw milk collected during one year from three groups of indigenous breed cattle: A-traditional households (n=650), B-microfarms (n=11) and C-commercial farms (n=2). Monthly there were analyzed every 16 samples of milk from each group summing a total of 320 samples per group, respectively 960 samples on the entire quantity of processed milk. Data interpretation was focused on the evaluation of the relevance of CFU and SCC values obtained from Ekoskope system tests, and also their statistical analysis in conjunction with seasonal influences. It was also analyzed the correlative evolution of framing these hygienic-sanitation parameters EU standards. From all outcome results, a particular relevance we attributed to the evolution of hygienic-sanitation parameters, which indicated important oscillations for SCC, with minimum values in summer (152300 cells/mL) and maximum in fall (240900 cells/mL) and statistically significant differences (p=0.0001). Regarding the growth of CFU, we noticed also important oscillations related with group and season. This parameter has reached higher levels (71210 CFU/mL) in the milk came from households or minimum values in summer (64300 CFU/mL) and the maximum in the autumn (76720 CFU/mL), with differences statistically significant (p=0.0001). Based on the results we appreciate the accuracy of conducted tests with automatic Ekoskope and Soleris systems and relevance statistically significant differences that indicate significant increases of SCC and CFU in autumn and reached minimum values in the summer. According to obtained data, these rapid testing cytological methods microbiological are relevant and opportune in the evaluation of the freshness and health in commodity milk as it allows immediate analysis of the correlation SCC-CFU in any processing units.

Key words: commodity milk, freshness, sanitation
The share of milk and milk products in human nutrition is growing because they contain most of macronutrients (proteins, lipids, carbohydrates) and micronutrients (vitamins and minerals) necessary for the growth and development of the body (16). In this context, ensuring the compositional and hygienic-sanitary milk quality are essential conditions for framing within quality standards of any dairy products quality standards of any dairy product (6). Current monitoring programs of hygienic-sanitary indices of commodity milk are focused on implementing fast methods of cytological and microbiological evaluation directly in the processing units. Such procedures are increasingly based on automated methods, among which highly topical are the ones based on utilising the Soleris and Ekoskope type systems. Analysis of data obtained using these automated systems are based on quantification of cellular content of milk, as the total number of somatic cells (NCS), respectively on microbial content, as the total number of germs (NTG). They also represent the basic indicators used in monitoring the health of the mammary gland in cattle and other animal species (5).

SCC is influenced by different factors linked to animal and environment, the major impact occurring to the species, milk yield, stage of lactation or to management practices (11). In spite of all oscillations that may present, SCC remains a key indicator for assessing the quality of milk from cow, goat and sheep (4,13). In this context we recall that, according to EU standards, SCC values for cow's milk currently have the following meanings: for individual cases, the increases over 200000 cel./mL typically indicates the evolution of a form of mastitis and over 400000 cel./mL mixing milk infers that it is unfit for human consumption.

SCC includes apart from normal micro flora components in milk also possible pathogenic microorganisms, major impacy on public health having the genera: Pseudomonas, Listeria, Salmonella, Escherichia, Staphylococcus, Clostridium, Bacillus or Vibrio (3,17). By action of own micro flora, the milk becomes an environment prone to fermentation transformations and decay of its components, some desired, used in industrial processing, and other pests that can remove the product from the consumer.

According to some studies based on an overview of the hygienic-health parameters of raw milk, 10L of pathological milk, with high residue content or inhibitory substances may compromise, during processing, up to 86000 L of normal milk, which once again confirms the major impact of mammary gland health upon the quality and health of the dairy utilised.

Framed within the context of things mentioned, this study involves the implementation in the processing unit of some rapid methods of evaluating the hygienic-sanitary parameters of commodity milk and establishing the correlation SCC-NTG necessary for monitoring the health of commodity milk.

Materials and method

The research we have conducted in this study consisted of assessing the relevance of automatic systems of Ekoskope and Soleris type in the cytological
(SCC) and microbiological (NTG) evaluation of commodity milk processed in a trading company from Gurghiului Valley. The tests were performed on samples of raw milk collected during a year, from cows of indigenous breed, organized in 3 groups: A-traditional households (n=650), B-micro farms (n=11) and C-commercial farms (n=2) (Table 1). Monthly, there were analysed 16 milk samples from each group totalling to 320 samples per group, namely 960 per the whole amount of milk processed. The average amount of milk supplied daily by the components of the three milk sources, was 24.6 L/individual producer, 909 L/micro farm and 3000 L/big farm. Thus, out of 32000 L of milk processed daily, 16000 L were from households, 10000 L from micro farms and only 6,000 L from big farms (Table 1).

Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Source</th>
<th>Number (units)</th>
<th>Share (%)</th>
<th>Daily average (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Households</td>
<td>650</td>
<td>50.00</td>
<td>16000</td>
</tr>
<tr>
<td>2</td>
<td>Micro farms</td>
<td>11</td>
<td>31.25</td>
<td>10000</td>
</tr>
<tr>
<td>3</td>
<td>Large farms</td>
<td>2</td>
<td>18.75</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>662</td>
<td>100</td>
<td>32000</td>
</tr>
</tbody>
</table>

The small scale producers, with an average of 3 animals/household, represented the main source of supply of commodity milk. They produce the milk under traditional household conditions with predominantly manual milking, the percentage of those using mechanic milking equipment being insignificant. The micro farms in the area have an average of 79 cattle and have good hygienic conditions, being equipped with automatic milking systems and their own cooling tanks. In the area there are two large farms with an average of 200 cattle. They have excellent zoo-hygiene conditions and hygienic milking conditions, being equipped with automatic milking rooms (equipped with automatic Aquarius type systems for the signalling of mastitis).

In the area prevails the Romanian Spotted breed and its mestizos, which maintain an increased potential for disease resistance and a high level of adapting to climate and maintenance conditions from Gurghiului Valley area. Regarding the feed of lactating cows we mention that it was predominantly based on natural grazing during the summer, respectively on hay feeding, produced in this mountainous area, supplementing with concentrate feed (mostly maize) during the stabbing period.

The working protocol consisted of collecting the samples of milk, complying to the sanitation standards and measures. The milk samples were collected in
sterile containers of 60 mL (no added preservative), ensuring optimal temperature conditions (4°C). Cytological and microbiological assessments were performed within the first 2-4 hours of collection, in the factory laboratory of the studied company. Cytological and microbiological tests were determined using automated systems, Ekoskope for type determining the SCC, respectively Soleris to determine NTG (Fig. 1).

Fig. 1. Overview images of the automate systems Ekoskope and Soleris, supplemented with details on the processing the raw milk samples

Evaluations through Soleris method were based on the principle of automatic detection, consisting of reproducible enumeration, quick (for 12 hours at 32°C) and precise of NTG from raw milk samples (2 mL), seeded on specific culture environments, containing nutritious agar. The basis of this method is the monitoring of colour changes, given by the chemical characteristics of the culture environment in response to microbial activity. Data interpretation was focused on assessing the relevance of NTG and SCC values obtained from automated tests, respectively from their statistical analysis correlated with seasonal influences. It
was also analysed the correlative evolution of framing these hygienic-sanitary parameters into EU standards.

Statistical analyses were based on statistical processing of data obtained using the MedCalc program, destined for statistics in biomedical research, respectively Graph Pad Prisma. Analysis of descriptive data and the distribution of variables were performed through summary statistic and frequency analysis. The graphic representation of registered data was based on the use of diagrams of “box plot” type. In these charts, each "box" represents the values between quartiles 25-75% of the values of the series and the line in each "box" represents the median of the group. Meanwhile, the lines that extend each "box" (vertical or horizontal), indicate the extent of the values in the first and fourth quartile. There were also used ANOVA and Bonferroni's Multiple Comparison and Chi-square tests, which allowed the comparison of the difference between the mean values of the three samples, p<0.05 indicating the statistical differences between groups.

Results and discussions

Analysis of the results obtained from cytological testing through the Ekoskope method. Benchmarking of data recorded during cytological tests of samples of raw milk with automatic Ekoskope machine focused mainly on the sample variables of season and provenance. They also showed significant oscillations of the SCC, but still complying with the admitted standards for consumption milk. Among them, noteworthy are the slightly higher average values (195700 cells/mL) for the sample A, milk originated from households, compared with the samples B (186200 cells/ml) and C (182500 cells/mL). Also to be noted are the statistically insignificant differences (p=0.07) recorded between these samples (Table 2). The overall analysis of the three sources of commodity milk of data obtained from benchmarking of the seasonal dynamics of the SCC revealed minimal values (152300 cells/mL) in summer and the maximum values in autumn (at 240900 cells/mL), with statistically significant differences (p=0.0001), indicating this parameter’s framing within normal range (Figure 2).

After synthesizing the values recorded after the cytological tests, one can see the framing of the individual and mean values of SCC within the admitted standards for commodity milk, with significant oscillations influenced by the source of the samples and thus by season. Within the admitted ranges were also framed the highest values of SCC (195700 cells/mL), obtained in the case of milk from small producers, also noting that the differences recorded between samples were statistically insignificant (p=0.07). In line with expectations, the seasonal development of SCC revealed minimal values (152300 cells/mL) during summer and maximum values during autumn (240900 cells/mL), with statistically significant differences (p=0.0001). References to such developments also make other relevant studies according to which SCC levels do not differ significantly, not even when comparing commodity milk obtained in the conventional system with milk obtained under ecological conditions (1). On the other hand, lower SCC values
obtained for the milk coming from large farms reveals the special conditions of hygiene, of maintenance and fodder they ensure. To those conditions it can be added the efficiency of computerized surveillance system, equipped with equipment which automatically signals mastitis.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Households</th>
<th>Micro farms</th>
<th>Large farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>231</td>
<td>233</td>
<td>237</td>
</tr>
<tr>
<td>Minimum</td>
<td>97.00</td>
<td>99.00</td>
<td>64.00</td>
</tr>
<tr>
<td>Median</td>
<td>189.0</td>
<td>188.0</td>
<td>168.0</td>
</tr>
<tr>
<td>Maximum</td>
<td>666.0</td>
<td>341.0</td>
<td>341.0</td>
</tr>
<tr>
<td>Mean</td>
<td>195.7</td>
<td>186.2</td>
<td>182.5</td>
</tr>
<tr>
<td>Standard Dev.</td>
<td>74.72</td>
<td>53.78</td>
<td>60.25</td>
</tr>
</tbody>
</table>

Fig. 2. The graphical representation at a group and season level of the evolution of average values of SCC from commodity milk

Analysis of the results obtained from microbiological testing through Soleris method. The correlative evaluation on groups of origin and seasons, of samples of commodity milk revealed important oscillations of NTG, which evolved to admissible standards for commodity milk (Table 3). Thus, in the case of the investigated samples were recorded the following variations: 71210 UFC/mL for the milk from households (group A); 56950 UFC/mL for those coming from micro farms (group B) and 50570 UFC/mL for milk from large farms (group C). As reveals the
data presented in Figure 3, the values of this parameter had oscillations grouped in the middle of the accepted standards, but with statistically significant differences between groups A and B, respectively A and C (p=0.0001). Regarding the seasonal comparative evolution of NTG from commodity milk, originated from the three sources, we found minimal values (64300 UFC/mL) in summer and maximum values in the autumn (76720 UFC/mL), the differences also being statistically significant (Fig. 3).

### Table 3

Variations of average values of ntg (ufc/ml) in the three sources of commodity milk

<table>
<thead>
<tr>
<th></th>
<th>Households</th>
<th>Microfarms</th>
<th>Large farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>227</td>
<td>233</td>
<td>237</td>
</tr>
<tr>
<td>Minimum</td>
<td>30.00</td>
<td>16.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Median</td>
<td>73.00</td>
<td>59.00</td>
<td>51.00</td>
</tr>
<tr>
<td>Maximum</td>
<td>99.00</td>
<td>87.00</td>
<td>99.00</td>
</tr>
<tr>
<td>Mean</td>
<td>71.21</td>
<td>56.95</td>
<td>50.57</td>
</tr>
<tr>
<td>Standard Dev.</td>
<td>12.79</td>
<td>14.08</td>
<td>17.27</td>
</tr>
</tbody>
</table>

Synthesis of data recorded for the analysis of values obtained after microbiological tests revealed significant oscillations of NTG, which was influenced by the variable sample and by the variable season. In this regard we only mention that the highest levels of NTG were found for samples of milk from small producers, between minimum values recorded in summer and the maximum values recorded in autumn, the differences being statistically significant (p=0.0001). Referring to these developments we point out that there are studies that indicate
increased levels of NTG in milk from traditional households of raising lactating cows (10). Moreover, other studies have demonstrated that milk obtained through conventional conditions may contain fewer germs than the one processed in ecological conditions, without such differences being biologically relevant (15). It is also to be noted that in the case of cattle breeding in traditional households, the system provides for the organization of milk collection centres, in which the milk stored in cooling tanks continues to be collected only once a day. Under these circumstances, the milk is kept overnight at temperatures of 2-4°C, time during which there is a considerable multiplication of the microbial population, with quantitative oscillations from one sample to another or from one season to another.

The overall analysis of all the results obtained from cytological and microbiological testing of milk samples studied showed their inclusion in national and European standards for raw milk. Currently, it is widely accepted the enormous impact of hygienic-health indices in controlling the health and quality of commodity milk, the supervision of dairy processing and the ensuring of dairy products’ food safety (7). Maintaining hygienic-health indices within compliance standards requires the introduction of automatic systems for monitoring breast health, based on the evaluation of the electrical conductivity of milk or its cellular content (8). In contrast, low levels of SCC in summer reveals reduced risk of mammary gland infection due to higher levels of hygiene and walk, conferred by natural grazing, especially in the case of mountain areas (18). Furthermore, administration of feed around milking time forces the cows to remain in quadrupedal position at least 60-90 minutes, which provides good protection against microbial invasion of the mammary gland. Poor outcome may have the cases with symptoms of clinical mastitis, but with negative results in laboratory testing. Such developments can have multiple explanations, including limited resources for the multiplication of the microbes producing the mastitis or the involvement of pathogens less known, usually non-bacterial. Mastitis are considered the most important pathogenic states with direct impact on the dairy industry and significant economic losses (9). Solving these problems requires good management of prevention and control against mastitis, leading to improved health of the mammary gland and ensuring a substantial reduction in direct costs and economic losses produced by the evolution of mammary infections in herds of lactating cows (2).

According to the phrase "SCC friend or foe", increasingly present in milk production and industrialization field (14), increased levels of SCC usually must be correlated with the development of mastitis in herds of lactating cows and by default with the affecting of hygienic-health and composition parameters of raw milk and processed dairy products (13). On the other hand, research in this area reveals that an increased number of cells in milk may influence, through their endogenous enzymes, the composition and even the technological properties of milk products (12, 14).
Conclusions

The results obtained in study show the relevance and benefits of using, at a level of processing unit, the Ekoskope and Soleris type automated systems in testing the main hygienic-health indices with a major impact in monitoring the health and quality of commodity milk. Evaluation of data resulting from NTG-SCC correlation, established on the basis of statistical analysis, indicated significant increases in these parameters in autumn and attainment of minimal values during summer.

Synthesizing the values recorded from cytological and microbiological analyses, carried out on a significant number of samples of collected milk reveals the support of implementing the automated Ekoskope and Soleris methods in as many processing units in order to rapidly assess the hygienic-sanitary quality of commodity milk.

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EFFECTS OF COCONUT WATER AND UHT SKIM MILK BASED EXTENDERS ON THE RAM SPERM PARAMETERS

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Summary

The aim of the study was to evaluate the effect of coconut water and UHT skim milk based extender and on the viability of ram semen stored at 4°C for 72 hours. Ejaculates were collected from three sexually mature and healthy adult rams. The semen was collected during the breeding season using electroejaculation procedure. After macroscopic and computer assisted sperm analysis (CASA, Microptic, Barcelona) the samples were diluted (1:2, 1:5 and 1:10) with coconut water based extender supplemented with 1% antibiotic antimycotic 100x (Sigma-Aldrich) and UHT skim milk. The motility and vitality were established for each dilution and the diluted samples were stored at 4°C. After 24, 48 and 72 h the samples were evaluated with CASA system. Coconut water showed a positive effect on ram semen, total and progressive motility and viability was higher for samples processed with coconut water (p <0.05).

Key words: ram, semen, coconut water, skim milk

Sperm long-term storage and cryopreservation, an important tool of artificial insemination, are the most efficient methods for sperm conservation in order to preserve the genetics of valuable, geographically isolated males (Holt WV. 2000, Gadani B et al., 2017). Semen cryopreservation induces structural alteration as results of thermal, mechanical, chemical osmotic and oxidative stress (Holt et al., 1992, Reda et al., 2016, Woelders et al., 1997, Tekin et al., 2006). These changes decrease sperm motility, vitality, acrosome integrity, plasma membrane functionality and as such fertilizing capacity (Reda et al., 2016). Therefore, the viability of sperm for a long period required specific and suitable extender in order to maintain the quality of spermatozoa (Daramola et al., 2016). Numerous methods have been used for assessment of the semen quality (motility and morphology) in various breeds (O’Conner et al., 1981, Mostafapor et al., 2014) and study in order to modify semen extenders for protecting sperm during freeze-thawing process (Forouzanfar et al. 2010, Emamverdi et al., 2013 ). Some natural products have been used as components of semen extenders due to their high content of polyphenol, flavonoid, vitamins and minerals etc (Rozkot et al., 2013, Tedeschi et al., 2004, Akandi et al., 2015). The purpose of this study was to evaluate different sperm parameters of ram sperm stored at 4°C temperature in different extenders containing coconut water and skim milk.
Materials and methods

Animals were anaesthetized with xylazine (0.2 mg/kg) and ketamine hydrochloride (1.4 mg/kg). Ejaculation was performed using a manual electroejaculator standardized for small ruminants (Minitube-Germany) consisting of a rectal probe 2 cm in diameter and 30 cm in length. The semen was collected into a graduated sterile conical centrifugation tube. Seven ejaculates were collected from three sexually mature and healthy adult rams. In each ejaculate, volume, concentration, sperm motility, vitality and morphology were assessed after collection.

Triladyl® (Minitube, Germany) was used as base and control extender. Only samples with sperm motility ≥ 80% were used in this study. The extenders were supplemented with different concentrations of coconut water and UHT skim milk (1:2, 1:5 and 1:10, commercial products) and 1% antibiotic antimycotic 100x (Sigma-Aldrich). The motility and vitality were established for each dilution and the diluted samples were stored at 4°C. After 24, 48 and 72 h the samples were evaluated with CASA system.

The semen parameters were assessed using a CASA system (Microptic, Barcelona, Spain). In order to assess sperm motility, aliquot semen was diluted in phosphate buffered saline supplemented with 1.5% BSA. A 3 µl of samples were transferred into a Leja counting chamber with the chamber depths of 20 µm. Progressive motility (PR) (rapid progressive, type A, medium progressive, type B), non-progressive motility (NP), immotile sperm (M) were evaluated. Sperm vitality was evaluated using FluoVit kit (Microptic, Barcelona, Spain) and CASA system. The morphology was determined using Diff-Quik® staining technique, acrosomal modification were assessed using Spermac® staining (Minitube, Germany).

Data were analyzed using GraphPad Prism 6 software (Manual Graph Pad Prism 5.0, GraphPad Software). A value of $p < 0.05$ was considered statistically significant.

Results and discussions

Semen was obtained from three mature, healthy Merinos ram. The total volume of sperm samples obtained by electroejaculation was 1.4±0.46, the sperm concentration was $367204 \times 10^6$ spermatozoa/ mL, vitality 96.42±4.11, the morphological abnormality was 10.53±1.48 and the level of acrosome integrity was 98.85±4.05.
In order to evaluate the protective effect of some natural products, the commercial extender was diluted with UHT skim milk and coconut water. The effects of different concentrations of coconut water and skim milk in extenders are presented in Table 2.

<table>
<thead>
<tr>
<th>Extender</th>
<th>Dilution</th>
<th>Storage time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Coconut water</td>
<td>1:2</td>
<td>71.2±0.2</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>81.4±0.4</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>88.2±0.7</td>
</tr>
<tr>
<td>UHT skim milk</td>
<td>1:2</td>
<td>55.3±0.9</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>59.2±0.1</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>67.4±1.5</td>
</tr>
</tbody>
</table>

There were significant differences among two types of supplement (coconut water and skim milk). Coconut water in dilution of 1:5 and 1:10 showed a positive effect on ram semen, the evaluated parameters was higher for samples processed with coconut water (p <0.05) compared with skim milk. Triladyl® supplemented with coconut water provides more effective preservation. Sperm motility parameters were higher in semen diluted with coconut water compared with UHT skim milk. The percentage of motile spermatozoa was significantly greater in samples diluted in proportion 1:5 and 1:10 with coconut water. In contrast, no significant differences were observed between extenders (P > 0.05) for morphological abnormality after refrigeration, but the acrosome integrity was different between two groups.

A significant increase in the percentage of total motility was observed after refrigeration with coconut based extender (first group) compared with skim milk based extender (second group). After 24 h of refrigeration the total motility in the first group was 80.26±8.55 compared with the second group where the total motility after 24 h was 60.63±6.17. After 48 h the total motility in the first group was 79.43±7.73 compared with the second group where the total motility after 48h was 59.63±6.29. The results after 72 h was comparable, the total motility in the first group was higher compared with the second group, 75.5±6.47 vs. 56.33±5.61.
Table 3

Ram sperm vitality percentage in Triladyl® supplemented with different concentration of coconut water and UHT skim milk

<table>
<thead>
<tr>
<th>Extender</th>
<th>Dilution</th>
<th>Storage time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Coconut water</td>
<td>1:2</td>
<td>79.2±0.2</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>84.4±0.2</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>86.2±1.2</td>
</tr>
<tr>
<td>UHT skim milk</td>
<td>1:2</td>
<td>60.3±0.9</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>60.2±0.1</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>68.5±1.1</td>
</tr>
</tbody>
</table>

In the same consent, there were also significant differences among extenders in viability level as shown in Table 3. Coconut based extender showed the highest significant (P < 0.005) viability index. A non-significant difference was found between morphological changes. Our study concluded that the incorporation of different concentrations of the coconut water in extender increased sperm motility and viability. There were significant high sperm motility values when extender was diluted 1:10 with coconut water and with skim milk. The viability level was significantly higher in most coconut water concentrations than control. The supplementation of the commercial Triladyl® extender with coconut water showed the highest significant results concerning the sperm motility and viability.

These results are in agreement with Uchoa et al., 2002 (26), who reported that the coconut water can be used in extenders for dog semen, and increased spermatozoa motility and improved sperm quality. Also, Cardoso et al., 2003 (4), showed that coconut water in addition with 20% egg yolk can be used successfully in cryopreservation of canine semen. Various types of macromolecules added as component of extender may affect the sperm parameters during storage (1). The uses of organic compounds, vegetable or animal for semen dilution and conservation have been reported by other authors (27). Numerous studies used coconut water based extender to the refrigeration andcriopreservation of sheep, swine and bees semen (2, 14, 3, 11, 16). Through its content coconut water provides and to improve sperm viability and morphology. Commercial coconut (Cocos nucifera) water is the clear liquid and contains sugars, vitamins, minerals and amino acids (6, 30). Santi et al., 2007 (21) shows that coconuts also able to synthesize different antimicrobial peptides in their water, with diverse properties and mechanisms of actions. Coconut water has positive activity against human pathogenic bacteria and represents a potent source of biotechnological products (21). Milk extender provided more effective preservation of the acrosomal membrane after cryopreservation (7). Our results indicated similar results for both extenders without statistical differences. Evaluation of plasma and acrosomal membrane integrity is important once they are related to sperm fertilizing capacity (18, 22). Coconut water and UHT skim milk showed a positive effect on ram semen
acrosomal integrity. Ottini et al., 2010, demonstrated that filtered coconut water added to the freezing media increases osmolarity of freezing media. In conclusion, this study indicates that coconut water have a positive effect on spermatic parameters through its unique nutritive, antioxidant and antimicrobial property.

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MORPHOLOGY OF THE UROGENITAL ORGANS OF THE MALE OSTRICH (Struthio camelus)

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Summary
Fertility of the male ostrich plays an important role increasing the results of the artificial insemination process. The bodies of three adult male ostriches from the Zoo of Timișoara were dissected. The urogenital organs were identified, studied and compared with the organs of the domestic birds.

Key words: urogenital organs, ostrich, male.

Ostrich (Struthio camelus) is a member of ratite family and shares the order Struthioniformes with the emu, rheas and kiwi. Is flightless specie native from Africa (1). Since ancient time the ostrich was used by man in different purpose. The utilitarian, spiritual and decorative value of this bird has been exploiting through the centuries (2). The ostrich industry is better established in the southern hemisphere (3) and continues to play a very important role in the economy of South Africa (2). The fertility of the male ostrich is considered to be the key of the increased production, mainly the artificial insemination process (2, 5). Therefore the anatomy of the cloaca and the male reproductive tract must be well known (4, 6).

The aim of the study was to describe the morphological features of the urogenital organs and compare with the domestic birds, bringing a contribution to a currently poor aspect of the male urogenital tract.

Materials and method

Three adult male ostriches were obtained from the Zoo of Timișoara and used in this study. The cadavers were dissected by specific methods which interested the abdominal cavity and the cloaca.

The organs of the urogenital tract have been identified, studied and described. Pictures were made and morphological features of the organs established.

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Results and discussions

The cadavers were arranged in the dorsal decubitus and the dissection interested the skin and the abdominal musculature. The digestive organs have been removed, so the urinary and genital organs could be identified. The cloaca has been examined and dissected. The penis was isolated, described and pictures were made.

Kidneys (*Rens*) are elongated and lie against the synsacrum, situated in the same plan. Though are molded on the recesses of the hip bone are enveloped by the renal capsule and a large amount of fat. The cranial, middle and caudal parts are not distinctive as in domestic birds. The color is reddish-brown and presented an elastic consistency. They measured 22 cm in length and 3 cm in width. Renal sinus is absent (Fig. 1).

Testes (*Testis*) are suspended and attached cranially to the kidneys. Right testis is more cranial than the left testis. They have ellipsoidal aspect, soft consistency and a white-yellow color. The fibrous capsule is thin and elastic giving a smooth appearance to the surface of testis. The testicular venous plexus is well represented (Fig. 2).

Epididymis (*Epididymus*) is firmly attached to the dorso-medial border of testis (Fig. 2).

Deferent duct (*Ductus deferent*) is thin and sinuous, accompanies laterally the corresponding ureter to the cloaca, where it opens symmetrically in proctodeum.

Penis (*Penis*) is large and promiscuous. Its origin is on the base of the cloaca where the two erectile bodies are fused. It is slightly curved, presenting spongy structure. The length in the quiescent status measured 11-12 cm (Fig. 3, 4, 5).
Fig. 2. Testes and epididymis in ostrich
A. Left testis; a. Cranial extremity; b. Caudal extremity; c. Medial surface; d. Lateral surface; e. Free border; B. Left epididymis; A’. Right testis; a’. Cranial extremity; b’. Caudal extremity; c’. Medial surface; d’. Lateral surface; e’. Free border; B’. Left epididymis.

Fig. 3. Penis of the ostrich
1. Base of the phallus; 2. Ventral groove; Apex.
Fig. 4. Cloaca and phallus in ostrich
1. Dorsal labrum of the cloaca; 2. Glandular part; 3. Phallic groove;

Fig. 5. Cloaca in ostrich
1. Dorsal labrum; 1’. Ventral labrum; 2-2’. Lateral commisures;
3. Penis.
Conclusions

In the ostrich the kidneys differ significantly from those of birds. The cranial, middle and caudal parts of kidneys are absent. Renal capsule is smooth and surrounded by a large amount of fat. Right testis is more cranial than the left testis. The penis is protrusive and even in quiescent status the apex is located out the cloaca. Ventral groove is straight and not ellipsoidal as in palmipedes. The vent of cloaca is rounded, not horizontal like in domestic birds.

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References

HISTOPATHOLOGICAL ASSESSMENT OF SYSTEMIC PATHOLOGY WITH VIRAL ETIOLOGY IN TWO CAPTIVE REPTILES

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Summary
Over the last few years the frequency of having a reptile as a pet has considerably increased. However, the uncertain origin and the poor husbandry may result into high morbidity and mortality. One Burmese python and one Savannah monitor, both adults and coming from private owners, were submitted for post mortem investigations in our department. Gross lesions seen in the Savannah monitor were sero-sanguinolent effusion in the coelomic cavity, massive hemorrhage of the left fat pad - with traumatic involvement, hepatic degeneration, inflammatory pulmonary edema, coprostasis and circulatory modifications of the ovaries, death occurring from cardio-respiratory failure. Gross lesions encountered in the Burmese python consisted in sero-sanguinolent effusion in the coelomic cavity, ulcerative stomatitis, myocardial degeneration, granulomas in the liver and gastrointestinal tract, haemorrhagic necrotising oophoritis. Microscopically, the pathology of the death causing lesions in both cases was completed by identifying intracytoplasmic and intranuclear viral inclusions. The Savannah monitor exhibited severe steatosis, endocarditis, gout in liver, kidney and lung and intracytoplasmic and intranuclear viral inclusions in spleen, pancreas, myocardium, lung and brain with suspicion of Paramyxovirus and Adenovirus. The microscopic pathology seen in the python is dominated by granulomas with unstructured central necrosis areas and mixed peripheral mononuclear and heterophilic infiltrate within the heart, liver, lungs, spleen and gastrointestinal tract, with intranuclear and intracytoplasmic inclusions of the hepatocytes, attributable to Herpesvirus and Arenavirus. Key words: Savannah monitor, Burmese python, intranuclear and intracytoplasmic inclusions.

Captive reptiles are prone to a wide variety of pathological conditions, caused by etiological factors such as changes in their habitat, husbandry issues or stress induced by transport or handling, leading to a suppressed immune system and increases the risk of disease (15). Every year, in boas and pythons only, more than 200,000 animals are exported (8). This involves mixing species from different geographic regions within exotic animal collections, resulting in the risk of transmitting pathogens to new species. At the same time, non-compliance to the
quarantine measures or releasing of captive animals increases the risk of infection (3, 15).

Studies in captive reptiles have cited an extremely varied pathology encountered in these species, where the skin, the respiratory and the gastrointestinal systems exhibit the highest involvement. Etiology is represented by nutritional imbalances and infectious agents, most of them being saprophyte species, but if correlated with a compromised immunity can lead to different pathological conditions. The main lesions were circulatory (edema, hyperemia), pulmonary inflammations, including the presence of abscesses and granulomas, visceral necrosis, accompanied by various cellular infiltrates (4, 5, 13, 15).

Viral diseases in reptiles have expanded around 1976 with the identification and isolation of viruses in chelonians, crocodiles and squamates (9). In the case of viruses that develop inclusion bodies, the diagnosis of a viral disease along with the orientation toward a particular viral species can be made on the morphology and the microscopic pathology using the histopathological examination, correlating this information with the antemortem clinical signs (1).

Materials and methods

Two captive reptiles, one Savannah monitor (*Varanus exanthematicus*) and one Burmese python (*Python molurus bivittatus*) were submitted for post mortem investigations in the Pathological Anatomy Laboratory of the Faculty of Veterinary Medicine in Bucharest. Both animals were adult, they came from a private owner, and no clinical signs were noticed before death. For the two cases necropsic examinations were performed, together with cytological slides from parenchymatous organs, May-Grunwald Giemsa stained, as well as organ and tissue sampling for the histopathological examination. The latter was carried out after the fixation of the samples in either 10% formaldehyde or Carnoy solution, followed by paraffin embedding and sectioning at 4-6 μm, the stainings used being Hematoxylin-Eosin (HE) and Gomori methenamine silver stain.

Results and discussions

Concerning the Savannah monitor, the exterior examination revealed a distended abdomen. The absence of the terminal segment of the tail was noticed, as well as 2-3 cm² shedding areas on the distal extremities of the limbs, suggesting dysecdysis.

The opening of the coelomic cavity (Fig. 1) revealed the presence of a transparent red effusion (60 ml). The myocardium was pink and flaccid. The yellowish-white lungs expressed a white foamy fluid when sectioned (Fig. 4), characteristic for edema. The yellowish-gray liver (Fig. 3), extremely friable, showed subcapsular emphysema areas. The gallbladder was distended.
Fig. 1. Serosanguinolent effusion in the coelomic cavity, coprostasis, necrohemorrhagic cephalthritis, hemorrhagic left fat pad.

Fig. 2. Hemorrhagic left fat pad

Fig. 3. Hepatic lipidosis, subcapsular edema

Fig. 4. Pulmonary edema

Fig. 5. Intracytoplasmic viral inclusions in the pneumocytes (arrows). HE, 200x

Fig. 6. Intranuclear viral inclusions in neurons (arrows). HE, 1000x
The kidneys showed no macroscopic lesions, except for some declive miliary areas on their surface, with specific infarct morphology. The digestive tract did not show any lesions, except for the stomach, containing some wood fragments covered by a whitish mucus, and the terminal portion of the intestine, which exhibited massive coproprostasis.

The ovarian follicles, white to dark red, exhibited various stages of development, suggesting a hemorrhagic-necrotizing oophoritis. The left adipose pad showed multiple subcapsular hematomas (Fig. 2) (lesion attributed by the literature to the traumas produced by aggression between individuals of the same species, following the stress of captivity) (13). The right fat pad did not exhibit any lesions.

In the histopathological examination, the liver presented an altered architecture on a background of hepatosteatosis. Hepatocytes were either entirely deteriorated or the remaining cell nuclei were eccentrically compressed by lipid vacuoles. Intralesional polymorphic bacteria and uric acid deposits of varying sizes were observed at both the Hematoxylin Eosin and Gomori stains. Large intracytoplasmic amphophilic or slightly basophilic inclusions were observed in the hepatocytes.

The lung exhibited an extremely thick interstitial area, with an abundant mononuclear inflammatory infiltrate represented by lymphocytes and macrophages, along with numerous heterophils observed either occasionally or as a massive infiltrate. The intracytoplasmic inclusions (Fig. 5) that were observed within hepatocytes were amphophilic/basophilic and large. Similar to the liver, intralesional polymorphic bacteria have been observed within the lung. The respiratory ducts contained yellowish-oxyphilic proteic material containing rare heterophils, macrophages and desquamated pneumocytes – edema.

The myocardium revealed areas with oxyphile muscle fibers, with the loss of the contractile structure, specific to hyalinization. Also, a massive mononuclear cell infiltrate has been noted among the cardiac muscle fibers. The same infiltrate, along with many heterophils, was noticed under the endocardium. Intralesional polymorphic bacteria, including large intracytoplasmic amphophilic/basophilic inclusion bodies have been observed within myocardial cells.

The histopathological examination of the brain revealed a spongious structure of the nervous substance, with the presence of perineuronal hollow optic spaces, occasional neuronal vacuolisation, and the presence of 1-3 μm intranuclear oxyphilic inclusion bodies (Fig. 6).

Concerning the Burmese python, the exterior examination revealed two red-gray areas underneath the corneous layer, 1-2 cm in diameter, without loss of substance, one in the ventral region of the tail, and the other on the left side of the middle third of the trunk, lesions with possible traumatic etiology due to bedding or objects in the terrarium. The macroscopic examination of the oral cavity revealed three ulcerated areas (Fig. 7), two of them located on the palatine area, and the third on the middle left mandibular area, with a maximum diameter of 3 cm. The central area of the lesion was covered by a gray material, and a reddish streak was
noted peripherally. These lesions were associated with osteolysis and tooth extraction in the left maxillary lesion. The macroscopic diagnosis was deep ulcerative stomatitis, a lesion that was commonly encountered in herpesvirosis (9).

Opening the coelomic cavity revealed 150 ml of yellow-red liquid. The pericardial cavity also contained 30 ml of yellow-gray effusion (Fig. 8).

The heart was pink, irregular in appearance, of flaccid consistency, dystrophic, almost entirely covered by petechiae. The surface of the ventricular epicardium was covered by an adherent whitish deposit with a macroscopic appearance of fibrinous epicarditis.

The lung exhibited dark grey areas; the cut surface revealed a yellow-red liquid that coagulated.

Throughout the length of the digestive tract, nodular lesions varying in size, ranging from a few millimeters to 9 cm in diameter (Fig. 9), of tough consistency, have been observed. They were yellow-gray or red, and the cut section showed a gray, compact appearance. In some cases the nodules presented an ulcerated surface, covered by a gray deposit. Also, the entire length of the digestive tract mucosa showed erosions, ulcers, and hemorrhages of variable size. The macroscopic diagnosis was granulomatous gastritis and enteritis. The content of the digestive tract was reduced, gray, liquid, along with a transparent mucus. In the pyloric area, the lumen was almost obstructed by the nodular lesions described above, which merged, leading to the necrosis of that segment and of the proximal duodenal area. The inflammatory process included both the spleen and the pancreas, the gallbladder being the only organ that could be detached from the area.

The liver presented several spherical nodules, 1-2 mm to 5 cm in diameter (Fig. 10), of elastic consistency, pink-gray on section, with caseous whitish areas and red-black areas of necrosis and hemorrhage.

The kidneys did not exhibit any gross lesions.

The ovaries presented follicles at various developmental stages, white to red-black, indicating a hemorrhagic-necrotizing oophoritis.

From a histopathological point of view, the pulmonary parenchyma exhibited acute hemorrhage, edema (proteic material in the faveolar space), desquamation of necrotizing pneumocytes, in admixture with numerous inflammatory cells (lymphocytes, macrophages, heterophils). Hemorrhagic areas showed an expansion tendency, involving both airspace and interstitium. The edema of the faveolar space involved a homogeneous proteic material, sometimes granular or even fibrin. The interalveolar septa were thickened and infiltrated with inflammatory (mononuclear and heterophilic) cells and active fibroblasts. In the less affected territories, areas of compensatory emphysema was observed. Within the faveolar epithelium occasional intracytoplasmic oxyphilic inclusions were noticed. The histopathological diagnosis was interstitial pneumonia with viral inclusions, necrotic bronchopneumonia and hemorrhages as a complication of the primary lesion.

The heart exhibited numerous areas of necrosis along with a heterophilic and mononuclear cells infiltrate. The blood vessels showed ectasia. Within the
myocardium granulomas were observed, with an unstructured necrotic center, bordered by a heterophilic infiltrate and eccentrically surrounded by an area of epithelioid cells. The histopathological diagnosis was severe granulomatous myocarditis.

The liver presented degenerated and necrotic hepatocytes. Extensive areas of picnotic, oxyphilic (apoptotic) hepatocytes have been observed. Intracytoplasmic and intranuclear inclusion bodies have been observed, 1 to 3 microns in diameter, most likely of viral origin (Fig. 11, 12). Also, vacuoles were noticed in the cell cytoplasm, which suggested microvesicular hepatic lipidosis. Interstitial granulomas were present, with central necrosis, bounded by a heterophilic and a mononuclear infiltrate. In the sinusoidal capillaries, numerous heterophiles have been observed, characteristic for a septicemic state. The histopathological diagnosis was piogranulomatous hepatitis, probably developed on the background of a viral etiology hepatitis.

The kidneys exhibited a thickened, edematous capsule. Within the glomeruli, the filtration space was greatly enlarged, the mesangial cells were active, the glomerular capillaries showed hyperemia, and heterophils were occasionally present. The Bowman capsule was thickened. Isolated epithelial cell necrosis was observed, along with medium accumulation of hemoglobinic pigment in proximal tubules. Also, hyalin degeneration and tubular necrosis were observed. The distal tubules developed proteic cylinders and granular to vacuolar degeneration. The interstitium was infiltrated with heterophils and showed edematous areas. Intracytoplasmic inclusions were identified in nephrocytes. The histopathological diagnosis was chronic glomerulonephritis.

The spleen showed congestion. Necrotic foci bordered by heterophils and fine collagen fibers were observed.

In the stomach, the lamina presented hyperemia and a discrete mononuclear infiltrate.

The intestine presented a partially intact epithelium, with erosion areas. Perivascular edema associated with a heterophilic and mononuclear infiltrate, as well as fibrin, were observed. An intramural heterophilic infiltrate was noticed, along with lymphocytes, macrophages, necrotic areas and bacterial colonies. The associated lymphoid tissue showed hyperplasia. In the pancreas, extensive necrosis infiltrated with heterophils, macrophages, fibrin and coccoid bacteria were noticed.

The brain exhibited a spongy pattern of the white matter, with edema and demyelination. Axonal edema was observed, with occasional formation of digestion chambers.
Fig. 7. Ulcerative stomatitis

Fig. 8. Pericardial effusion, fibrinous epicarditis, myocardial degeneration.

Fig. 9. Granulomatous enteritis

Fig. 10. Granulomatous hepatitis, hepatic lipidosis

Fig. 11. Hepatic lipidosis; Intranuclear viral inclusions within hepatocytes (arrows). HE, 400x

Fig. 12. Hepatic lipidosis; Intracytoplasmic viral inclusions within hepatocytes (arrows). HE, 400x
Perineuronal and perivascular vacuoles, hyperemia, occasional picnosis and neuronal vacuolization - signs of hypoxia - were observed. In addition, lipofuscin granules and intranuclear and intracytoplasmic oxyphilic and amphophilic viral inclusions were observed in microglia and neurons.

There is a constant presence of inclusions in different cells in both studied cases, both intranuclear and intracytoplasmic. Viral infections that develop inclusion bodies are extensively studied in the literature.

Intranuclear inclusions in Lacertilia species were commonly found in herpesviruses, where in green iguana (Iguana iguana) the macroscopic examination did not reveal any lesions, but the histopathological exam revealed a lympho-histiocytic infiltrate in the liver, spleen, kidney and bone marrow. Also, in common agama (Agama agama) and San Esteban chuckwalla (Sauromalus varius), herpesviruses have been associated with visceral necrosis, pneumonia, with intranuclear inclusions in the liver, spleen and lung (10, 17).

Adenovirosis has been diagnosed in numerous reptile species. The reported lesions consisted of proliferation of the tracheal and esophageal mucosa in Jackson's chameleon (Chamaleo jacksoni), liver necrosis in the bearded dragon (Pogona vitticeps) and lympho-histiocytic infiltration, hepatitis and multifocal necrotic myocarditis in the savannah monitor (Varanus exanthematicus), the lesions being associated with the presence of either basophilic or eosinophilic intranuclear inclusions (9, 11, 13). In the above-mentioned savannah monitor, degeneration of cardiac muscle fibers was observed, as well as the presence of a massive mononuclear infiltration among the dilated cardiac muscle fibers. Viral oxyphilic intranuclear inclusions were observed within neurons, all of which advocated for a possible adenovirus diagnosis in this case.

Intranuclear inclusions were also found in parvovirosis. The main feature of replication of this virus is the dependence on cells with a high replication capacity, such as those of the intestinal tract or bone marrow. In bearded dragons (Pogona vitticeps) viral particles specific for parvovirosis and adenovirosis were identified in the intestine and liver (9).

Paramyxovirus develops inclusion bodies in the cytoplasm and nucleus. In caiman lizard (Dracaena guianensis) it was associated with pneumonia and caseous material in the lung, while microscopically a heterophilic and histiocytic pneumonia was observed (9, 13). The lesional aspects were similar to those seen in the monitor, such as an interstitial pneumonia with abundant inflammatory infiltrate, represented both by lymphocytes and macrophages, as well as by numerous heterophiles. Viral inclusions were intracytoplasmic, and observed mostly in pneumocytes.

Herpesvirosis in the Ophidia members (snakes) was cited in boa constrictor (Boa constrictor), for which mortality was recorded in juveniles younger than one year old, and histopathologically lesions such as hepatic necrosis, exudative glomerulonephritis and amphophilic intranuclear inclusions were frequently observed, or in Aesculapian snake (Elaphe longissima), the four-lined snake (Elaphe quatuorlineata), Gaboon viper (Bitis gabonica), and boa constrictor (Boa
constrictor), where the clinical signs consisted of stomatitis, lack of appetite, nervous signs, and the diagnosis revealed a variety of viruses, such as adeno-, parvo-, picornas- and herpes- (9). Gastroenteritis in herpesviruses in snakes was also cited (18). In horned viper (Vipera ammodytes) diagnosed with herpesvirus, which exhibited lethargy, anorexia and dyspnea, the macroscopic lesions consisted of hemorrhages, accumulation of sero-sanguinous effusions in the cavities, miliary white foci in the liver, while the microscopic ones were represented by coagulative necrosis in the liver, hyperemia and mononuclear infiltrate, to which eosinophilic intranuclear inclusions were added (2). The lesional aspects presented for the previously presented python are similar, with necrotic stomatitis, hemorrhages and intestinal necrosis accompanied by a heterophil-rich infiltrate, lymphocytes and macrophages, extensive liver necrosis, chronic glomerulonephritis, along with the presence of intranuclear viral inclusions, which suggest a possible involvement of this virus in the pathology of the case. Taking into account the staining variations of histopathologically dependent inclusions, as well as associations of several viral species or similar clinical signs, other viruses can not be excluded.

As with Lacertilia members, snake adenoviruses have been associated with hepatic necrosis, hepatomegaly, and basophilic intranuclear inclusions in constrictor boa (Boa constrictor). In Colubrids, sudden death was encountered, and the histopathological examination revealed necrotic lesions in the intestine, including intranuclear basophilic inclusions in enterocytes. In Viperids, the adenovirus has been associated with hemorrhages and ulcerations in the intestinal tract, or esophagitis and stomatitis, with intranuclear eosinophilic inclusions (9, 14). Parvovirus in snakes was associated with necrotic stomatitis, gastroenteritis, liver necrosis, pneumonia, lack of appetite, nervous signs, yellow feces. Microscopically, basophilic intranuclear inclusions were observed, and in addition to the parvovirus, adeno-, picornas- and herpesvirus were isolated (6, 9).

The paramyxovirus was cited in Viperidae, black mamba (Dendroaspis polylepis), ball python (Python regius) and Burmese python (Python mulurus bivittatus), where nervous signs and respiratory manifestations were noted. Microscopically, eosinophilic and basophilic intracytoplasmic inclusions were observed in pulmonary alveolar cells and glial cells in the central nervous system (9, 14). In another study, also in Viperidae, pulmonary signs were not quoted, but the nervous and digestive ones were present. Macroscopically, areas of pulmonary consolidation, with hemorrhages and exsudates, hepatic nodules, and interstitial pneumonia were observed, while microscopically piogranulomatous and necrotic hepatitis were diagnosed (10).

Inclusion Body Disease, recently classified as Arenavirus, has been diagnosed with captive snakes since 1970 with the help of specific intracytoplasmic inclusions. Generally, the virus is fatal due to overlapping infections which infected animals develop, but there are situations where the virus remains latent for prolonged periods (3, 8, 12). Nowadays many species of the virus are known in Boidae (1). The cited clinical signs are abnormal behavior, exhaustion, torticollis,
imbalance, opistotonus, impossibility of returning to physiological position after induced dorsal decubitus, regurgitation (3, 16). The lesions were represented by stomatitis, pneumonia, proliferative lesions, with large intracytoplasmic inclusions in the hepatocytes, respiratory epithelium, pancreatic acini, gastrointestinal epithelium, esophageal epithelium, epithelium of renal tubes (3). Arenavirus was also diagnosed in a captive boa constrictor (Boa constrictor) as a surprise diagnosis of a gingival fibromyxoma, both on the presence of specific intracytoplasmic inclusions in the histopathological examination and with RT-PCR (7). In the case of the python, the observed lesions can be correlated with this virosis. They consisted of stomatitis, interstitial pneumonia, degeneration of the nervous substance, all of which are supported by the presence of oxyphilic intracytoplasmic inclusions in the alveolar epithelium, in hepatocytes, nephrocytes and neurons.

Conclusions

In the Burmese python, correlating macroscopic and histopathological lesions, the intracytoplasmic inclusions can be attributed to Arenavirus, while the intranuclear inclusions may advocate for Herpesvirus.

In the Savannah monitor, the intranuclear inclusions plead for adenovirosis, while the intracytoplasmic ones, together with the gross lesions, could be attributed to a Paramyxovirus.

References


THE MORPHOLOGICAL PARTICULARITIES OF THE THORACIC LIMB BONES AT LAMA GLAMA (LINNAEUS, 1758) - CASE STUDY

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Summary
The present scientific paper aims to analyze and describe the morphological characteristics of the bones of the thoracic limb in llama (Lama glama) in order to provide new data in this species regarding the method of morphological recognition based on particularities of the skeletal system. Lama glama is a domesticated descendant of the Lama guanaco species, being widely encountered in Peru and in the North-Western part of Argentina. In Europe, Lama glama can be only encountered in zoos and circus shows. For this scientific paper, we used an individual adult llama that was donated to the Anatomy Department of the Faculty of Veterinary Medicine Bucharest, by the Circus & Variete Globus. The bones, described in this scientific paper, were obtained by removing the soft tissue, and after that they were subjected to a process of controlled maceration, washed and degreased. The description of the bones was done according to the Nomina Anatomica Veterinaria, 2005. The scapula is characterized by a very developed and obvious elongated acromion. At the proximal extremity of the humerus an intermediary tubercle can be observed, whereas distally the epicondyles do not outreach the caudal articular surface. The radius presents, at its distal extremity, an articular surface represented cranially by two glenoid cavities, and caudally by three condyles. The distal extremity of the ulna is completely integrated in the radius. There are seven carpal bones, disposed in two rows. The main metacarpal is formed through the welding of the third and fourth metacarpals, their distal extremities remaining separated through a deep intermetacarpal notch.

Key words: llama, thoracic limb, scapula, humerus, radius

Lama glama is a species belonging to the Lama genus, Camelidae family, family that includes the camel, the dromedary and three South American species: vicuna, alpaca and guanaco. The Lama glama is a domesticated descendant of the Lama guanaco (2,4,6) species encountered in Peru and North-West Argentina. In Romania, Lama glama can be found in the Bucharest-Baneasa Zoo as well as in circuses. There are relatively few data in the scientific literature regarding the morphology of the appendicular skeleton of this species, some scientific papers present mainly general characteristics of camelids rather than comparative data on the skeleton.(3,7,9) The present scientific study was done using the bones of an
adult *Lama glama* aiming to present certain particularities on which bones or fragments of bones can be differentiated from those of other species of herbivores (large ruminants and equines).

**Materials and methods**

The study material was an adult llama (*Lama glama*), dead of natural causes and donated to the Anatomy Department of Faculty of Veterinary Medicine by Circus&Variete Globus Bucharest. The bones of the thoracic limb were thoroughly cleaned of soft tissue and there were then subjected to a process of controlled maceration, cleaning and degreasing,(1,5,8) The maceration was done in containers kept at a constant temperature for a long time (approximately 50 days) under constant supervision. The cleaning was initially done under a continuous stream of water for 24-48 hours. After the maceration process, the bones were cleaned over again with the tip of the scalpel in order to remove all organic remnants. The degreasing was done using regular detergents diluted in the water used for cleaning. The cleaning was done with mildly sparkle water and any traces of organic origin were further removed. The drying was done under supervision for 48-56 hours at a temperature of 18-22°C in order to avoid the fissuring of the bone structures in order to not compromise their integrity. Measurements were made, and the most interesting aspects were described and photographed. The description, identification and homologation were done according to the *Nomina Anatomica Veterinaria* (N.A.V) 2005. The zoo technical compass, the ruler and the callipers were used for measurements.

**Results and discussions**

The scapula, a flat bone, has a length of approximately 26,1 cm from its dorsal edge to the glenoid angle, on the direction of the scapular spine, while the width measured on a perpendicular through the middle of the length is of 7,6 cm. The length/width report is of 3,43.

The dorsal edge, with a length of 18,2 cm has a rough surface on which the scapular cartilage inserts itself.

The cranial edge of the scapula, with a length of about 22 cm, is concave in its middle and distal thirds, and presents a wide scapular notch.

The caudal edge, with a length of 19,9 cm appears thickened in the middle third and flattened from front to back, being crossed on its surface by numerous muscular insertion lines.

The lateral side is divided in two fossae, supraspinous and infraspinous, in a report of 1:3. The scapular spine has a length of 23,4 cm, it begins non-evidently but finishes with a considerable acromion. The spine is rectilinear all throughout its length and presents a lengthened tuberosity in its middle third, with a length of 7,6 cm. The acromion has a length of approximately 2,5 cm (Fig.1).
Fig. 1. The scapula in llama (Lama glama) – lateral side (original)

Fig. 2. The scapula in llama (Lama glama) - distal extremity (original)
The medial side of the scapula presents in the proximal area hardly visible serrate surfaces. The subscapular fossa is wide and lengthened, having numerous muscular insertion lines on its surface. The neck of the scapula is well emphasized. The cervical angle is supple, while the thoracic angle is slightly thickened. The articular angle has a slightly oval glenoid cavity with a small glenoid notch, arranged cranio-medial.

The supraglenoid tuberosity detaches itself far away from the brow of the glenoid cavity and it is well outlined, looking rather oval shaped, with a length of 3,8 cm and a width of 3,5 cm. Cranio-medial, an emphasized and medial recurved coracoid process, with a length of 1,3 cm detaches from the supraglenoid tuberosity (Fig. 2).

The humerus has a length of 26,3 cm, and the width at the middle of its length (measured cranio-caudal) is of 3,1 cm. The length/width report is of 8,48. The articular head, slightly oriented cranio-medial is supported by a visible neck.

The great tubercle, disposed laterally in the proximal extremity, is divided into a cranial great tubercle, with a convex aspect and a length of 3,3 cm, and a reduced caudal great tubercle. The lesser tubercle, disposed medial, is also divided into a cranial lesser tubercle with an elongated aspect and a length of 2,7 cm and a reduced caudal lesser tubercle. Between the great and the lesser tubercles, an intermediary tubercle is present, which will divide the intertubercular groove in two tendinous slide grooves (Fig. 3).

The infraspinous surface is emphasized and circular shape.

The tubercle for the teres minor muscle is well distinguished. The anconeal crest is reduced in size with a length of 1,7 cm, while the deltoid crest is very evident, with a length of 2,7 cm. It continues distally until it nears the half of the diaphysis. In the medial third of the humeral body a nutrition hole can be observed disposed cranio-lateral.

On the medial side of the humeral body the tubercle for the insertion of the teres major muscle can be noticed. From this tubercle begin, medio-cranially, two lines for muscular insertion. The brachial groove is not evident. At its distal extremity, the humerus presents, as articular surface - a condyle, disposed laterally, with the length of 2,8 cm and a trochlea with irregular borders, disposed medial. The medial lip is much more developed, taller, with a sharp edge and a width of 2,8 cm, while the lateral only measures 0,8 cm. The epicondyles do not pass the distal articular surface. The radial fossae is elongated and shallow, while the olecranon fossae is deep and wide. The forelimb bones, represented by radius and ulna, are welded together, making two radio-ulnar arcades only in the proximal and distal extremities.
Fig. 3. The humerus in llama (*Lama glama*) (original)


The radius is a long bone, of 29.5 cm, and the width measured latero-medial at the half of the length is of 3.1 cm. The length/width report is of 9.51. The proximal extremity of the radius articulates synostosial with the ulna. Caudal, the articular surface is represented by two glenoid cavities, the medial one disposed slightly obliquely, medio-distal. On the cranial line of the proximal extremity a clear coronoid process can be observed.

The body of the radius is flattened cranio-caudal and is convex cranial, articulating with the ulna synostosial, forming two reduced radio-ulnar arcades, a proximal one and a distal one. On the cranial surface of the distal extremity of the body, two clear tendinous slide grooves can be observed, disposed longitudinal, and a third more reduced one disposed slightly obliquely, medio-ventral (Fig. 4).
Fig. 4. Radius and ulna in llama (Lama glama) - cranial side (original)

Fig. 5. Radius and ulna in llama (Lama glama) - proximal extremity (original)

On the lateral side of the proximal extremity of the radial body, an emphasized lateral tuberosity can be observed, while medial a reduced rugose fossae is present.

The distal extremity includes an articular surface, represented in the cranial area by two glenoid cavities and in the caudal area by three condyles, from which the lateral one belongs to the ulna that welds completely, at this level, to the radius.

The ulna presents a smooth olecranon, with a length of 6.7 cm. The olecranon tuberosity is clear and cranial; it is crossed by a reduced transverse groove. The anconeal process is drawn cranial, while the semilunar notch continues the articular surface of the medial glenoid cavity of the radius (Fig. 5).

The body of the ulna reduces gradually in the middle third, being articulated synostosial in radius, in order to become obvious in the distal third, forming a reduced distal radio-ulnar arcade. The distal extremity of the ulna is completely integrated in the radius. The total length of the ulna is 36 cm.

There are 7 carpal bones, disposed in two rows (Fig. 6). The proximal row presents, in lateral-medial way the following bones: the accessory carpal bone, flattened lateral-medial, having a shape of a boot which peak is oriented dorsal, presenting two articular surfaces: a dorsal one for the dorsal extremity condyle of the ulna and a ventral one for the ulnar carpal bone. The ulnar carpal bone has its dorsal side with a slightly excavated articular surface in the radius, and its ventral
side relatively excavated in the fourth carpal bone. The medial side of the ulnar
carpal bone has articular surfaces for the accessory carpal bone. On the proximal
side, a reduced tubercle is present, and on the lateral side the entire surface is
rough. The intermediate carpal bone has strangulation in its central region, and on
the caudal-ventral side it presents a clear tubercle, with a tendency to become
tricuspid. The distal articular surface is divided by a medial relief in two relatively
equal portions. The radial carpal bone placed medial, has a rather discoidal shape,
having rough and not articular medial, proximal and distal surfaces. The dorsal
articular surface is strongly excavated, while the ventral one is divided by a relief,
into two articular surfaces disposed on different levels.

Fig. 6. Carpal bones in llama (Lama glama) (original)
1. Accessory carpal bone; 2. Ulnar carpal bone; 3. Intermediate carpal bone;
4. Radial carpal bone; 5. Fourth carpal bone; 6. Third carpal bone 7. Second carpal
bone

The second row of carpal bones, on the lateral-medial side, is represented
by the fourth carpal bone, the third carpal bone and the second carpal bone.

The fourth carpal bone, flattened on the dorsal-ventral side, has a relatively
flat articular surface, disposed on the ventral side, while on the dorsal side the
articular surface is divided by a reduced relief into two unequal parts, the one for
the ulnar carpal bone being more developed compared to the one for the
intermediate carpal bone. On the caudal-ventral side, the fourth carpal bone has an
clear tubercle.

The third carpal bone is flattened on the dorsal-ventral side, with its dorsal
articular surface divided by a relief into two surfaces, the one for the radial carpal
bone being more developed than the one for the intermediate carpal bone. The
ventral articular surface is relatively flat and continuous, but on the caudal – ventral
side it presents a clear tubercule.

The second carpal bone is reduced, with a dorsal articular surface that is
convex in every way, and a plane ventral articular surface.
The main metacarpal bone, with a length of 22 cm and a diameter of 2.6 cm was formed through the fusion of the III and IV metacarpals. This fusion is incomplete at the distal extremity where every participating metacarpal can be observed.

At the proximal extremity, the articular surface of the carpal bones from the second row is wide, the one on the medial side is rather flat, and the one on the lateral side is divided by a relief in two unequal parts.

At the proximal extremity of the body of the metacarpal, cranial-medial, there is a tuberosity for muscular insertions.

The cranial face of the main metacarpal is convex, while on its surface we can observe a reduced notch, which is the welding spot of the two metacarpals for the III and IV fingers. On the palmar side there is also a longitudinal groove which is very well emphasized in the middle third of the main metacarpal. This groove disappears progressively towards the distal extremity (Fig. 7).

In the third distal extremity of the body the two metacarpals, III and IV, are completely divided, each of them presenting an articular surface for the proximal phalange and for the great sesamoids.

The distal extremity of each metacarpal is represented by a surface with a condyle shape, disposed cranial, while on the palmar side there are two condyles divided by a median relief.

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**Fig. 7. Metacarpal in llama (Lama glama) (original)**


The thoracic acropodium is made of three phalanges at each finger (III, IV) and 4 great sesamoids (Fig. 8). The proximal phalanx is a long bone, with a length of 8 cm and a diameter of 1,5, with a cylindroids shape, whose proximal extremity presents a slightly excavated articular surface, with a semi-circular shape. In the posterior side, a central placed fossae can be observed. On the palmar side, in the proximal extremity, both lateral-palmar and medial-palmar, on the perimeter of the articular surface there is a reduced tubercle. At the proximal extremity of the bone body, on the palmar side, two rough ligament insertion surfaces can be observed, having a relatively triangular shape and the apex oriented in a distal way.

At the distal extremity, on the cranial side, there is an articular surface of condyle type, and on the palmar side, there are two condyles, divided by a reduced median groove.

The medium phalanx, with a length of 3,9 cm and a diameter of 1,8, has, on the proximal side, an articular surface with the shape of a cavity, and on the distal side, it has two condyles divided by a reduced median groove.

The distal phalanx, with a length of 2,4 cm, shaped like a triangular pyramid has a concave articular surface and three sides: abaxial, axial and solear. On the axial side, a vascular hole can be observed near the articular edge.

The great sesamoids, four in number, present a curved cranial side and a slightly concave articular side in the dorsal-vental side. The lesser sesamoid is not present; it is possible to have been integrated to the distal phalanx during this evolution of species.

![Fig. 8. The thoracic acropodium in llama (Lama glama) (original)](image1)
1. Proximal phalanx; 2. Medial phalanx; 3. Distal phalanx

![Fig. 9. The proximal phalanx in llama (Lama glama) - palmar side (original)](image2)
1. Tubercles for ligament insertion; 2. Surfaces for ligament insertion; 3. Distal extremity (trochlea)
Conclusions

The scapula in llama is similar to the one in the small ruminant, presenting the scapular spine as rectilinear, with an elongated tuberosity of the spine and a very well developed elongated acromion. Medially, at the level of the subscapular fossae, numerous muscular insertion lines can be noticed. The supraglenoid tuberosity detaches far away from the brow of the glenoid cavity, and the coracoid process is very well developed.

The humerus has an intermediary tubercle disposed between the two cranial tubercles, delimiting two tendinous slide grooves. The tubercle for the teres minor muscle is very well developed, and the deltoid crest is obvious. In the middle third of the body of the humerus nutrition hole can be observed disposed cranio-lateral. The medial side is marked by two lines for muscular insertion which begins distal in a cranio-medial direction from the tubercle for the teres major muscle.

The radius has the anterior side of the diaphysis convex both transversal and proximo-distal. At the proximal end there are two glenoid cavities and distal, the articular surface is represented by two glenoid cavities disposed cranial as well as three condyles, the lateral one belonging to the distal extremity of the ulna. The semilunar notch has an articular surface continuing the one of the medial glenoid cavity.

There are 7 carpal bones. The accessory carpal bone has the shape of a boot with two articular surfaces. The ulnar carpal bone has a slightly excavated dorsal side and on the proximal side it presents a small tubercle. The intermediate carpal bone bone has strangulation in its central region, and on the caudal-ventral side it presents a clear tubercle, with a tendency to become tricuspid. The radial carpal bone has a relatively discoidal shape, with a strongly excavated articular surface. The fourth carpal bone has a clear caudal – ventral tubercle. The third carpal bone is flattened on the dorsal-ventral side and the second carpal bone is reduced, with the dorsal articular surface that is convex in every way.

The main metacarpal was formed through the fusion of the III and IV metacarpals. The fusion is incomplete in the distal extremity of the main metacarpal, where each participating metacarpals can be observed. On the palmar side, a wide and deep groove can be observed, being very well shaped in the third middle of the main metacarpal, while in the distal extremity it totally disappears. The distal extremity of each metacarpal is represented by a condyle shape surface, placed on the dorsal side, and on the palmar side by two condyles divided by a median relief.

The proximal phalanx has, on its palmar side, in the proximal extremity, a each reduced tubercle and two rough ligament insertion surfaces with a relatively triangular shape, having the apex faced down.

In the distal extremity, on the cranial side, a condyle type of articular surface can be observed, and on the palmar side, we can observe two condyles, divided by a small median groove. The distal phalanx has the shape of a triangular pyramid. The great sesamoids are present while the lesser sesamoid is absent.
References

Summary
The aim of this study was to assess the cellular stress induced by hexavalent chromium exposure on rat's pancreas and the possibility to reduce its effects by administration of Aronia melanocarpa aqueous extract. In this purpose the expression of Bcl2 and Bax genes was determined by PCR analysis. The results pointed out that hexavalent chromium induces apoptosis in rats pancreas cells. The cellular damage was reduced in groups receiving the plant extract and thus highlighting the beneficial influence of Aronia melanocarpa aqueous extract on hexavalent chromium toxicity.

Key words: Aronia melanocarpa, pancreas, apoptosis, hexavalent chromium

The interest in dietary cyto-protective effects against oxidative stress is increasing day by day. Natural antioxidants have been used in therapy and prophylaxis of oxidative stress induced by free radicals (11). Oxidative stress appears when body's antioxidant capacity is overrun by the accumulation of reactive oxygen species. In high amount the ROS are toxic and affect cellular integrity, which leads to organ damage (16). Increased ROS levels are entailed in cellular injuries like oxidative damage, cell cycle arrest and apoptosis (24). Likewise ROS can induce the activation of pro-apoptotic signaling pathways – endoplasmic reticulum stress and mitochondrial dysfunction – resulting cell apoptosis (6). Apoptosis is a programmed cell death and can be induced by different exogenous chemicals. It manifests with cytoplasm shrinkage, chromatin condensation and DNA fragmentation (14). Caspase family proteins, Bcl-2 family members, are used as determinants of apoptosis as well as other biomarkers and signaling pathways (5). The Bcl-2 protein family is represented by pro-apoptotic and anti-apoptotic proteins, which are regulators and effectors of mitochondrial apoptosis pathway. Expression and activation levels of Bcl-2 proteins that occur during response to stress or death signals will determine the cell to coerce or stimulate cell death (29).

Aronia melanocarpa, from Rosaceae family, also called black chokeberry is a shrub native from North America, but nowadays cultivated in Europe extensively (3). The composition of this plant is responsible for its wide medicinal and therapeutic uses. The popularity Aronia gained in recent years is due to its content in polyphenols. Namely, Aronia is rich in anthocyanins, proanthocyanidins,
flavonols and hydroxycinnamic acids. Because of this content Aronia has the highest antioxidant activity among all berries (30). Also, the studies have demonstrated anti-viral, anti-mutagenic, anti-inflammatory, gastroprotective, hepatoprotective and anti-diabetic properties of Aronia extract or juice (4, 12, 13).

In the environment chromium appears mainly in two oxidations states: trivalent and hexavalent (17). More than 50 industries worldwide represent source of hexavalent chromium contamination. Chromium is non-biodegradable and persists in the environment. Hexavalent form of this metal is toxic and listed by the International Agency for Research on Cancer (IARC) in Group 1 as human carcinogens (10). It is known that Cr VI can enter rapidly into the cell, using sulfate anion channels, and it undergoes reductive reactions resulting intermediate forms of chromium (Cr V and Cr IV) and final Cr III. During this reduction the reactive oxygen species (ROS) are formed. The resulting chromium intermediate and ROS are responsible for chromium cytotoxicity (9, 17).

Therefore, the aim of this study was: a) to see if hexavalent chromium administration induces apoptosis in pancreas cells by assessment of Bcl-2 genes family expression, and b) to test Aronia melanocarpa ability to protect the pancreas cells from oxidative stress induced by hexavalent chromium.

Materials and methods

Experimental model

For this study were used 35 adult male Wistar rats (220–240g) that were purchased from a licensed breeder. The animals were divided in five groups:

- Cr group - treated with a hexavalent chromium compound, potassium dichromate, for three months;
- CrA group – treated with hexavalent chromium and Aronia melanocarpa aqueous extract for three months;
- Cr2 group – treated with hexavalent chromium for three months and that one more month with distilled water only;
- CrA2 group – treated with hexavalent chromium for three months and one more only with Aronia melanocarpa aqueous extract, and
- control group (C) which received distilled water.

Cr VI was administered in distilled water at dose at 75 ppm, level that represents 3x LOAEL (27). Our previous studies (18-23, 26) showed that at this dose damages were the most severe.

The rats were housed under standard laboratory conditions: 12 h light/12 h dark cycle, 25±2°C temperature, food and water ad libitum, according to the guide for laboratory animals care and use.

Aronia melanocarpa 2.5 % aqueous extract was obtained by classic extraction: dried berries – purchased from natural plant shop – were mixed with distilled water, ratio of 0.25/10 water/volume, and then heated at 90°C for 10 minutes. The extract resulted after filtering the heated mixture (1).
At the end of exposure period, the rats were sacrificed following the national and international law regarding the care and use of laboratory animals.

**qRT-PCR Analysis**

The sample of 50g from pancreas was used for total RNA isolation and purification by SV Total RNA Isolation System (Promega), following the manufacturer’s protocol. Spectrophotometric method was applied on a NanoDrop8000 spectrophotometer (Thermo Scientific) for quality and quantity determination of purified RNA.

Then the cDNA was synthesized from 2µl of total RNA with First cDNA Synthesis Kit (Thermo Scientific). The 150ng of cDNA represented template for qPCR analysis. GoTaq qPCR Master Mix Kit (Promega) using the MX 3000P real-time PCR system (Agilent Technologies) was performed for gene expression analysis. Primers used in this experiment are presented in the table below. For normalizing the reactions GAPDH gene expression was used. All results were interpreted by 2-ΔΔC(T) method.

Primers used in this experiment are:

- **Sense (5'-3')**
  - GAPDH - ATGGAGAAGGCTGGGGCTCACCT
  - Bax - CCAGGACGATCCACCAAGAAGC
  - Bcl 2 - GGATGACTTCTCTCGTCTAGCTACCT

- **Antisense (5'-3')**
  - GAPDH - AGCCCTTCCACGATGCCAAAGTTGT
  - Bax - TGCCACACGGAAGAAGACCTCTCG
  - Bcl 2 - ATCCCTGAAGAGTTCCTCCACCAC

**Statistical analysis**

The results were statistically analyzed using one-way ANOVA, Stata 13 program (StataCorp LP, Texas, USA). Values of P < 0.05 were considered statistically significant.

**Results and discussions**

Apoptosis is a process controlled by genes, the most important ones being the members of Bcl2 gene family. Bcl2 is anti-apoptotic gene with the capacity to block apoptotic pathways. The opposite of Bcl2 is a pro-apoptotic Bax gene, with the ability to stimulate apoptosis (28). The products of these genes can interact and form homo- and heterodimers. Bax protein formation of homodimers in the outer mitochondrial membrane is responsible for pro-apoptotic action (25). On the other hand Bcl2 possess the ability to form with Bax heterodimers, which in turn prevent homodimer formation. Thus the Bcl2/Bax ratio is considered to be a key factor in regulation of apoptosis (8).

Expression of Bax gene increased very highly statistically significant in all experimental groups compared to the control one (C).
When hexavalent chromium was administered concomitant with aqueous extract of *Aronia melanocarpa* (CrA) very highly significant decrease was observed in the expression level of Bax gene compared to the group that received only chromium. In CrA group expression of this gene was the closest to the value registered in C group, but it was still very highly significantly increased which highlights the chromium induced stress in pancreas cells.

In the Cr² group, treated with hexavalent chromium for three months followed by one month administration of distilled water only the expression level of Bax gene was the highest when compared to other experimental groups. Compared to Cr and CrA group the increase was statistically very highly significant.

Administration of *Aronia melanocarpa* extract for one month after three months of Cr VI exposure reveals highly significant increase in level of expression when compared to Cr and CrA groups.

Expression of anti-apoptotic Bcl2 gene presents the same evolution as Bax gene since its role is block the initiation of apoptotic cascade by suppressing pro-apoptotic activity of the Bax gene expression.

The path that the cell will take is closely correlated with Bax/Bcl2 ratio. If this value is high the cell death will occur, but if this ratio is low than the cells can resist to apoptotic stimuli. The Bax/Bcl2 ratios has the following values: Cr² Bax/Bcl2 – 4.10; Cr Bax/Bcl2 – 3.86; CrA Bax/Bcl2 – 1.6; CrA² Bax/Bcl2 – 0.62 (Fig. 1).

Overexpression of Bcl2 and Bax gene, with Bax/Bcl2 ratio in favor to Bax gene, as observed in Cr² group, highlights the initiation of apoptotic cascade by overcoming cells adaptation mechanisms.

It was observed in the Cr group also, the overexpression of Bax and Bcl2 proteins, the Bax/Bcl2 ratio being 3.86, which represents the Cr VI action as a stress factor inducing the apoptotic mechanisms.

Hexavalent chromium induced apoptotic process can be due to the chromium possibility to bind and interact with cellular DNA, causing its damage by ROS production or by direct alteration like modification of nitrogenous bases, catena breaks, and formation of Cr-DNA adducts (7). Banu et al. (2) demonstrated that Cr VI administration induces apoptosis in granulosa cells by attenuating anti-apoptotic pathways (Bcl2) and stabilizing pro-apoptotic proteins (Bax), releasing the cytochrome c into cytoplasm by increasing the permeability of mitochondrial membrane. Likewise, Zhang et al. (31) showed that Cr VI induces apoptosis in human hepatocytes by ER stress and mitochondria pathways.
The mitochondria are organelles with antioxidant defense system which maintains redox balance in the cell. And as such it represents the main source of ROS (15).

In CrA and CrA2 groups the Bax/Bcl2 ratio value is around 1, meaning that administration of *Aronia melanocarpa* 2.5 % aqueous extract concomitant with Cr VI or alone in recovery period time of one month has blocked the initiation of apoptosis mechanisms in pancreas cells. The results are sustained by the other studies in which Cr VI induced apoptosis was diminished by administration of oxidative stress reducing agents like: vitamin C (2) and curcumin (15).

**Conclusions**

The results obtained in this study pointed out that Cr VI induces apoptosis in pancreas cells, by activation of mitochondria pathways. *Aronia melanocarpa* extract had the beneficial effect on pancreas cells, reducing the gene level expression of Bcl2 family when administered concomitantly with Cr VI or alone in recovery period time (one month). It can be concluded that *Aronia melanocarpa* extract has the ability to reduce the Cr VI induced activation of apoptosis cascade.
Acknowledgements

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OBSERVATIONS OF ISOFLURANE ANESTHESIA IN BEES (APIS MELLIFERA)

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Summary
The study was conducted on ten groups of worker bees exposed for 15 minutes at a concentration of 1.2 isoflurane MAC (L1-L5 groups) and 1.5 MAC (L6-L10 groups) in 1 liter / minute oxygen flow. During the induction period and awakening from anesthesia was observed bees behavior, and was noted the lifetime of bees which were held forwards captive. Narcosis induction, to all individuals and all groups, went quickly and quietly in minutes. Any bee had not died. During awakening period climbing reflex returns to all individuals and social behavior seems to not be influenced by anesthesia. The life expectation of bees monitored in this study fits in that estimated for spring and summer and has not increased due to isolation.

Key words: bees, isoflurane, social behavior, lifespan.

The growth and healthcare of invertebrates is the preserve of hobbyists and less of the veterinarians and the information regarding bees’ anesthesia are found in journals belonging to non-veterinary disciplines.

Why is needed anesthesia in the case of bees? Anesthesia is a necessary bad when queens are marked, during the artificial insemination, in order to immobilize for sampling, for examination under the microscope, in order to bring together hives, during swarming and when a new queen is introduced. The bee has become a unique model through which were tested the effects of anesthesia on the time perception and the circadian rhythm with important conclusions for human medicine (5), for studies related to learning and memory (6, 13, 14). The bee can become, like all insects, model for assessing the potential of anesthetic substances in mammals because the power of the majority of anesthetics differ only slightly between different species and even different phyla (1). Over time were recorded interesting observations on a wide range of anesthesia consequences, for example narcosis induced by cooling affect the function of olfactory memory in relation to the acquisition and consolidation of information while the remembrance or the information recovery appears to be resistant to chill (6), chilling of bees may cause retrograde amnesia and affects memory formation whether it is carried out within five minutes after the learning episode or conditioning (8, 9). Exposure to carbon dioxide induces permanent changes in foraging activity according to some authors, (2, 10, 18), while other researches argue the absence of any negative
Influences on behavior (16). As well, is advanced the idea that the development of internal organs is inhibited in worker bees (2, 10, 16, 18) and that the survival rate and the speed of awakening is dependent on the concentration of the oxygen-nitrogen to which they are exposed during post anesthetic period (11).

The isoflurane is probably the agent choice for terrestrial invertebrate’s anesthesia. It has been described its use in millipedes, spiders and nematodes (7). References regarding its use in bees are few.

In this paper we proposed to monitor the relevant anesthetics events during the fifteen minutes of isoflurane anesthesia and in the immediate wake-up period, the bee survival and their life expectance.

Materials and methods

Inhalation anesthesia was performed in semi-open respiratory system. A plastic container that is sealed with a lid at the top (anesthesia chamber) and having the dimensions of 17 cm / 11 cm / 11 cm (2057 cm$^3$) was attached through a connector to the gas analyzer (Datex Capnomac Ultima) and anesthesia machine (fig. 1a). At the opposite end, open to the room, was attached a charcoal filter to prevent environmental pollution (fig. 1b, c).

![Fig. 1. Anesthesia machine, anesthesia chamber and gas analyzer (a), the attachment to gas analyzer and to anesthesia machine (b), active carbon filter attached to anesthesia chamber (c)](image)

The study was conducted on ten groups of worker bees aged between 1-2 days, five groups were exposed for 15 minutes at a concentration of 1.2 iso-MAC (L1-L5 groups) and another five to 1.5 iso-MAC (L6-L10 groups) in 1 liter / minute oxygen flow. Each group has included ten bees for an easier handling and observation. Before of anesthesia, all the bees had free access to food. During isoflurane exposure were recorded:
- time interval to immobility installation, as a marker of anesthesia, for the first and last bee in each group;
- the behavior during induction period;
- the occurrence of accidents such intra-anesthetic death or other events.
After fifteen minute of isoflurane anesthesia bees were moved into another container, prepared in advance, provided with holes for air circulation, hole to evacuate the bodies of died bees and having a honeycomb (fig. 2). During the wake-up period was recorded the time elapsed until:
- the first reflex - abdominal movements, movements of the limbs, the first movements of antennas;
- the bees start to go individually, in other words, the first steps;
- the first successful escalation of container wall or honeycomb;
- when all the bees are awake.

![Fig. 2. Containers where bees were held captive until death](image)

After the complete recovery of all bees, the influence of anesthesia on social behavior was observed, recording if bees:
- stay isolated - none at a distance equal to the body size;
- are in intimate contact and touching each other.

Captive bees were fed daily. Water was also changed daily. In similar conditions was cared a control group (LM) which has not been anesthetized. It was recorded daily number of dead bees and the time elapsed to the death of the last bee in each group.

Was compared the cumulative number of deaths in three time periods:
- the first 0-7 days;
- 8-14 days;
- and at more than 15 days after anesthesia.

Was noted with TS$_{50}$ the survival time expressed in the number of days elapsed until when 50% of the bees of each group die.

**Results and discussions**

The behavior during the induction period is described below and the moments of narcosis beginning, respectively the immobility as a marker of anesthesia, at all ten groups are shown in figure 3.
In the groups L1-L5, to which anesthesia was done performed at a concentration of 1.2 iso-MAC, the immobilization of the first bee occurred after 1:42 minutes and last bee remained motionless after 3:51 minutes. The induction of anesthesia is calm, most bees adopt abdominal decubitus. In every group just one to two bees of all ten bees are supine (fig. 4) During induction only a small number of bees (1-4) remain hanging on the wall, but later fall to the container floor where they remain in abdominal position. Mandibles tremor was present in approximately 75% of individuals of each group. Fine movements of the antenna remained present in all bees. There were no deaths recorded during anesthesia.

Fig. 3. Installation time of the immobility (the minute) - narcosis - after initiation of exposure to isoflurane

Fig. 4. Bees position during 1.2 iso-MAC in first 2 minutes in group L2 (a) and L5 (b)
Immobility occurrence at first bee in groups L6-L10 was noted at minute 1:42, the last bee became motionless at minute 3:28. All bees remained in abdominal decubitus, completely motionless without limb or antennae movements, these appear in response to external stimuli the type of knocking the wall. During induction few bees (six of the fifty) remained quiet but attached for a long time to the container wall. There was no dead bee.

The return of the first reflexes after isoflurane shutdown, namely the first steps of bees belonging to L1-L5 groups were recorded at minute 2, the climbing on honeycomb held at minute 2:30. After 6 minutes, more than 2/3 of bees in each group were already climbed on the wall or honeycomb. To about 7 minutes the bees were already awake, few (one or two) already were eating. At 10 minutes all the bees were active (fig. 5). Evaluation of social behavior on full awakening reveals that nearly half of entire number of bees in each group were alone, the other half being in contact. Among bees found on honeycomb, vast majority already were eating, and only just one or two were not interested by food.

First steps of bee belonging to L6-L10 groups appear at minute 3 and the first obvious, vigorous movements of antennas and limbs at minute 1:30. The climbing on honeycomb appears shortly, namely at minute 3:30. All bees are awake after 10:15 minutes and they have a tendency of climbing the wall and honeycomb, where they begin to feed. Of the ten bees in each group, without exception, after complete awakening alone are only two or three, the rest are in intimate contact.

![Graph](image)

**Fig. 5. Events during recovery from anesthesia**

After 3 minute of carbon dioxide anesthesia, carried out in boxes of 7 cm / 11 cm / 6 cm, followed by one minute exposure to the different proportions of the oxygen-nitrogen mixture, the first movements of bees were observed between 4-12 minutes post anesthetic, and bees awakening occurred within 8-18 minutes (12).
These results are close to those obtained in this study when bees were exposed to concentrations of 1.2 or 1.5 iso-MAC.

Isoflurane anesthesia is running calm, are still present antennas movements and trembling of the mandible only when lower isoflurane concentration are used, and finally the bees anesthetized with isoflurane remain motionless in decubital abdominal position. This behavior is different from that seen in halothane anesthesia, in which bees are agitated with uncoordinated movements and shake of legs, with outward of proboscis and regurgitations, with erratically flight, bending of the abdomen, with temporary aggression episodes and needle externalizing (15) or with those in which ether is used when in the first 2-4 minutes of exposure the aggressiveness is exacerbated and is followed by a gradual mollification but the lowest stimulus will excite them vigorously (4).

The social behavior of bees to full awakening does not seem to be very different between groups. Analyzing the data obtained shows that at least half of the bees are grouped, meaning that are in intimate contact and either they are eating or are commencing cleaning activity of their legs, wings and trunk. In other words show normal behavior. Neither isolated bees, namely at a distance greater than the length of the body to the other bees, not seem to have behavioral changes that reflect side effects of anesthesia. They are moving quiet and balanced without tendency to fly, or keep a position of normal state. In none of the groups, was not showed the tendency to resume the flight or normal hive buzzing.

Climbing reflex used to assess motor function and performance primarily in experiments on flies (Drosophila melanogaster) anesthetized with carbon dioxide, returns to all bees after awakening. Anesthesia with carbon dioxide performed on flies, has been shown to reduce the ability of climbing correlated with the dose and duration of exposure (3). In spite of prolonged exposure for 15 minutes, and different anesthetic concentration, changes in motor function were not found through monitoring this reflex.

The review of cumulative deaths on time intervals is shown in table 1. The analysis of data from monitoring shows that the lowest number of deaths was recorded in all groups during the first two intervals. With the passage in the third surveillance interval, the number of deaths increased, accounting over 70% respectively 80% of deaths in two groups and over 90% in the remainder groups.

TS50, having a value of 32 days was equal in eight of monitored groups, namely the groups L1-L4, L7, L9, and LM. In the group L5 and L6 the value was less with four days, meaning 28 days and in the group L10 of 29 days (fig. 6). In other words in seven groups, half of bees were died at the same time with those of control group.

The increase of mortality dependent of age is considered intimately linked to foraging activity and with the accidents that may occur associated with it. The hypothesis that through removal of extrinsic mortality factors is reduced the mortality of bees isolated in a confined space, was not confirmed (17). Regarding the study of bees longevity after anesthesia, it was demonstrated that after carbon dioxide anesthesia lifespan expectation significantly decreases whether anesthesia
is single or repeated (19). The life of monitored bees (maximum 38 days in group L3, minimum 34 days in L1 and 37 days in group LM) in this study fall within that estimated for spring and summer, five to six weeks, in opinion of some authors even seven (20, 21, 22), without any influence due to anesthesia or of the protection against external factors of mortality.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>L6</th>
<th>L7</th>
<th>L8</th>
<th>L9</th>
<th>L10</th>
<th>LM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time interval 0-7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≥ 15</td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>8</td>
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<td>9</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Time limit in which has been registered the death of half of the bees (TS$_{50}$) in each group

**Conclusions**

The induction of general anesthesia, at all bees and all groups, is quickly and quietly in few minutes.

No differences were observed between groups in terms of social behavior of bees to full awakening.

Climbing reflex used to assess motor function, returns to all bees after awakening.

During isoflurane anesthesia to no group no death was recorded.
The life of bees monitored in this study fall within the estimated limits for spring and summer. There was not found a reduction in life expectancy as a result of the anesthesia.

There was no increase in the life expectancy as a result of protection (isolation of the bees) against external mortality factors.

Acknowledgements

This study was realised using the support and infrastructure project "Dezvoltarea infrastructurii de cercetare, educație și servicii în domeniile medicinii veterinare și tehnologiilor inovative pentru RO 05", cod SMIS-CSNR 2669.

References

IN-DEPTH ANALYSIS ABOUT BRUCELLOSIS IN CENTER-SOUTHERN SICILY

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Summary

Brucellosis of ruminants is a serious problem in Sicily and, therefore, also human brucellosis is a medical emergency. In the last years Sicily issued eradication plans increasingly persuasive, with the aim to eradicate definitely brucellosis from the region. These plans were designed to assist in all activities already set out under a thorough study of the area, identifying problem areas and farms which are particularly at risk for what concerned the spread of brucellosis, to carry out in-depth epidemiological investigations and define additional activities aimed to isolation and characterization of biovars of Brucella spp. circulating in infected farms on the island. The authors have carried out in the course of the years between 2013 and 2016 about 140 inspections, in the infected farms and in slaughterhouses where seropositive subjects were slaughtered, aimed at understanding the causes of the presence of the disease in these farms and obtaining different types of sample in order to isolate and subsequently characterize the different biovars of Brucella spp. circulating in the territory. The results of these activities are reported in this paper.

Key words: brucellosis, eradication program

Brucellosis is a worldwide bacterial zoonosis, due to gram-negative bacteria belonging to genus Brucella. It represents a serious problem for public health, causing a lot of economic losses due to reduced performances in the farms and depending on human disease management. In the most part of Italy it was eradicated after the adoption of a national eradication plan in the 90’s, but in the south of Italy and, particularly, in Sicily brucellosis is endemic and is still present. All the districts of Sicilian region are infected, although with different prevalence. In the last years National and Regional Health Administration issued eradication plans increasingly persuasive with the aim to eradicate definitely brucellosis from the region. These plans provide for a reduction of the number of days included between notification of the presence of infection in a farm and slaughtering of positive animals; then serological controls in infected farms must be done with a shorter frequency to remove infected subjects as fast as possible and finally it was decided to intensify the surveillance at the slaughterhouse, taking samples from infected animals to isolate Brucella, to characterize every site and, if necessary, to use the strains isolated to detect the origin of the infection itself using biotechnological methods. In 2015, with the new ordinance (4), it was provided a closer and more useful interaction between laboratory and veterinary public...
services, to achieve in-depth epidemiological survey with the aim at understanding the reasons both of the presence and of the persistence of brucellosis in some areas. The study reports the results of field activities carried out during three years 2013-2016 in the Sicilian districts localized in the center-southern Sicily: Caltanissetta and Enna. During these years the authors, which work in Area Caltanissetta of Istituto Zooprofilattico Sperimentale of Sicily, carried out 143 inspections both in slaughterhouses and in infected farms.

**Materials and methods**

In the period between March 2013 and December 2016 were carried out 143 inspections both in infected farms and at slaughterhouse in the districts of competence (table 1). At first the inspections aimed to obtain samples to isolate *Brucella spp.*; they were represented by milk and vaginal swabs, if taken in the farm from alive infected animals, or by lymph nodes, breast, spleen, uterus or testes if sampled at slaughter. Samples, after a stomacher digestion (if required), were inoculated into *Brucella* broth and incubated at 37°C in a 5% CO2 atmosphere for six weeks. Every week a sample of *Brucella* broth was inoculated into *Brucella* agar plates and incubated as described above. Growth was evaluated every eight days and typical colonies of *Brucella spp.* were examined according to the method described in the OIE Manual (2008). Biovar typing was carried out at the National Brucellosis Reference Center. From July 2015 the inspections in infected farms, carried out together with Veterinary services, in addition to the other activities provided in-depth epidemiological surveys to understand the origin of the infection.

<table>
<thead>
<tr>
<th>Inspections carried out per year</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>District</td>
<td>2013</td>
<td>2014</td>
<td>2015</td>
<td>2016</td>
</tr>
<tr>
<td>Caltanissetta</td>
<td>37</td>
<td>6</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Enna</td>
<td>22</td>
<td>8</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>totale</td>
<td>59</td>
<td>14</td>
<td>24</td>
<td>46</td>
</tr>
</tbody>
</table>

**Results and discussions**

During these 143 inspections, 378 samples were taken from serologically positive animals and from 113 was isolated *Brucella spp.* Strain typing carried out at the National Brucellosis Reference Center showed that all the isolated strains obtained from sheep and goats were *Brucella melitensis* biovar 3, while the ones obtained from cattle were mostly *Brucella abortus* biovar 3 and sometimes *Brucella melitensis* biovar 3, as reported in other studies (2, 3). From a valuation of all the epidemiological surveys, collected during last 18 months, it appears that the
presence of Brucellosis in the considered area is due to the specific type of husbandries(1): the habit of moving animals towards pasture using communal paths, shared pastures, lack of fences to defend pasture and water from unwanted contacts and, at least, lack of control about reproductive activities of the cattle. Sometimes the farmer doesn't know if an animal had an abortion or wasn't pregnant at all.

Conclusions

Surely all the activities carried out in these years reduced both the prevalence and the incidence of brucellosis in the considered districts, but we have still lot to do to eradicate this problematic infection. Probably it would be necessary to emphasize to the farmers, in every infected farms, that good husbandry and management systems are indispensable to reduce and erase the infection.

References

RADIOGRAPHIC MEASUREMENTS OF HOOVES IN HORSES WITH LAMENESS

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Summary
The study was conducted on 18 horses, including 14 horses that presented lameness. Radiographic measurements are the most common means to appraise the changes that develop inside horses hoof. Therefore, radiographic measurements provide clues to evaluate the quality of trimming, the development of some pathologic processes and the position of the third phalanx in relation to the structures from within the hoof. One consequence of changes in angles inside the hoof will be the increase of the pressure on the deep digital flexor tendon on phalanx III and on the sole, which causes the lameness. This changes of the angles are met in laminitis and navicular syndrome. Angle measurements are useful to both veterinarians, as well as farriers to establish a diagnosis for the lameness and to perform orthopedic shoeing in order to balance the horses’s hooves.

Key word: hoof, radiographic image, angles, lameness, horse

The pathological changes that occur on the surface of the hoof are a cause of lameness in the work, leisure and sport horses. These changes have been associated to a poor conformation and improper hoof balance. The conformation of the hoof is described by angle measurements and by the size of the hoof box, by the size of the third phalanx, and the relationship between them.

The value of the hoof angle given by the dorsal edge and the ground is of 45°-50° for the thoracic limbs and of 50°-55° for the hind limbs. There must exist a parallelism between the dorsal side of the wall and the dorsal side of the third phalanx, so that the angle formed by the dorsal side of the third phalanx and the ground should be equal to the angle which was presented before. The palmar angle, situated between the edge of the underside of the third phalanx and a parallel line to the ground must not exceed 10°, or have a negative value (12).

A too large angle made by the dorsal wall and the ground will cause some musculoskeletal injuries (III metacarpus fractures, humerus, disinsertion of ligaments, tendinitis). Also, a reduction of this angle and the angle formed by the distal phalanx and the ground increases the tension on the digital flexor tendon which will cause compression on the navicular bone (14,8).

The reduction of the hoof angle occurs as a result of lowering the heels during trimming or as a consequence of improper shoeing by lifting the hoof’s forehead, increasing the tension on the deep digital flexor, and opposed to it,
lowering the hoof's forehead and rising the heels by shoeing will decrease the tension on the deep digital flexor tendon (1). Following the studies of Reeden (2010), due to increased pressure because of the deep digital flexor tendon on the navicular bone and bursa there will appear irreversible changes locally as osteolysis and the fracture of the navicular bone, osteoarthritis, tendinitis of the deep digital flexor all of these leading to the occurrence of lameness (11).

Lameness in horses with navicular syndrome occurs in thoracic limbs and has an insidious debut, and in some cases lameness disappears when the animal is heated. In time, however, lameness becomes excessive even during exercise. The animal takes a short step and rests on the hoof's forehead, and lameness is usually bilateral. Hard surfaces and walking in closed circles will make lameness worse. Also, the shape of the horn box shows changes at the horses with navicular syndrome, such as short and narrow hoofs with high heels (8, 10).

An important measurement is given by the distance between the dorsal wall and the third phalanx, both at the proximal and distal level, which together with the rotation angle of the third phalanx (the angle formed by a parallel line with the dorsal side of the wall and the dorsal edge of the third phalanx), shows the rotation angle in laminitis, as a result of the detachment between the dermal and the epidermal lamellae. In laminitis, as a result of the detachment of the lamellae and the dermal lamellae from the epidermal ones, there occurs the rotation of the third phalanx, and so, the surface of the dorsal edge of the hoof and that of the third phalanx won't be parallel anymore, the distances measured at the proximal and distal level of the two won't be equal, and the rotation angle varies depending on the tilt degree which has a variation between 5° - 25° degrees and if there is no intervention, there will be a penetration of the sole by the third phalanx (4, 12).

In horses with laminitis there may appear mild forms of lameness which evolves until the animal is unable to move and takes a characteristic position "camped before and underneath him". Locally, there can be noticed increased heart rate on the digital arteries and the appearance of the disease is related to the administered food (excessive intake of carbohydrates, green food), sudden temperature changes, metabolic disorders (hypothyroidism, metabolic syndrome equine), age (often between 4 and 10 years), and is more frequent in females (4).

A hoof is trimmed correctly when the dorsal surface of the wall and the surface of the fetlock are on the same level and have the same alignment. The studies conducted on horses which had pressure sensors at the hoof level, had shown that the forces which were acting on the heels during walking, trotting and galloping were low when the dorsal surface of the wall and the region of the fetlock were in the same plane, and in that case the horses did not present lameness (12).

The evaluation and radiographic interpretation is being done carefully because the measurements are influenced by factors such as trimming, the surface of the paddock and the exercises. In order to interpret radiographic images regarding changes in bone structure and the relationship between angles, it is important to know the normal radiographic appearance. The results of the hoof measurements can be used for the prevention and treatment of lameness (14).
The study was conducted in order to compare the parameters obtained from the hooves of the horses that showed lameness and from those which didn't.

**Materials and methods**

The study was conducted on a number of 18 horses aged 1 to 18 years old. The horses have been clinically examined in order to determine the location and degree of lameness. To establish the localization on the lameness, the following tests have been conducted:

- interphalangeal extension test;
- interphalangeal flexion test
- testing deep sensitivity in the hoof using pliers to try hoof.

In order to establish the degree of lameness, it was taken into consideration the AAEP system (American Association of Equine Practitioners) lameness scale.* Table 1, used by the veterinarians from the USA to differentiate the degree of lameness depending on the animal's behavior while standing, stepping and trotting (9).

Before the hoof x-ray was performed, the hoof was cleansed and the horseshoe was removed from the clinically healthy horses and from those who had reacted positively following the application of the hoof test, showing the symptoms of a navicular syndrom.

Table 1

<table>
<thead>
<tr>
<th>AAEP (American Association of Equine Practitioners) lameness scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grade of Lameness</strong></td>
</tr>
<tr>
<td>Grade 0</td>
</tr>
<tr>
<td>Grade 1</td>
</tr>
<tr>
<td>Grade 2</td>
</tr>
<tr>
<td>Grade 3</td>
</tr>
<tr>
<td>Grade 4</td>
</tr>
<tr>
<td>Grade 5</td>
</tr>
</tbody>
</table>

By using the grading system of the degree of lameness "AAEP lameness scale."(8) of the 18 examined horses of the following was observed:
• 4 horses with grade 0 of lameness;
• 1 horse with one degree of lameness;
• 5 horses with grade 2 of lameness;
• 4 horses with grade 3 of lameness;
• 3 horses with grade 4 of lameness;
• 1 horse with grade 5 of lameness.

Radiographs were performed with the Siemens Multix Swing using the following parameters: 70-75 kV and 20-25 mAs for exposure. The incidence used was the latero-medial one to track the position of the third phalanx in the horn box, the X-ray beam was centered halfway between the dorsal and the palmar side of the coronary region.

The following angles were taken into consideration:
• the angle of the hoof formed between the dorsal edge of the wall and the ground;
• the angle of the third phalanx, formed between the dorsal edge of the third phalanx and the ground;
• the palmar angle, formed between the ventral edge of the third phalanx and a line parallel to the ground

Angle measurement was performed using the classical reporter and the Carestream Image Suite Veterinary Software Angle Measurement Software.

Results and discussions

The results obtained are noted in Table 2 and show the dimension of the angles that have been measured at the hoof’s level. There are differences in the value of the angles obtained at the horses that didn’t present lameness in comparison to those which did.

Out of the examined horses, 4 horses were clinically healthy, 10 horses were diagnosed with navicular syndrome and the remaining 4 with laminitis.

There are also differences between the dimensions of the angles obtained from horses with navicular syndrome versus those diagnosed with laminitis as well as the degrees of lameness, which were much more serious in the case of laminitis. In horses diagnosed with navicular syndrome (5-14), lameness was ranked in Grade 2 and Grade 3, and for horses diagnosed with laminitis (15-18) ranked in grade 4 and 5 lameness.

At the non-lameness horses (Fig. 1), the size of the hoof angle is within the normal parameters, having an average of 54.25 and a standard deviation of 2.5 while the horses which presented lameness and navicular syndrome have
recorded higher values, having an average of 61.75 and a standard deviation of 4.65, and in those diagnosed with laminitis (Fig.2) the average is 59.33 and the standard deviation of 11.58. This standard deviation observed on horses which presented laminitis shows a high variation among the group as a result of the horn box growth, met by Hertsch B, Teschner D., in a study conducted on 117 horses (6).

At the non-lameness horses, the size of the third phalanx angle falls into normal parameters with an average value of 52.25 and a standard deviation of 3.3, while the horses which presented lameness and were diagnosed with navicular syndrome (Fig.3) had higher values and an average of 59 and a standard deviation of 4.8, and those which were diagnosed with laminitis had an average of 64.66 and the standard deviation of 1.3.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/ Years</th>
<th>Breed</th>
<th>Size of angle</th>
<th>Lameness</th>
<th>Grade of lameness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hoof angle</td>
<td>Third phalanx angle</td>
<td>Palmar angle</td>
</tr>
<tr>
<td>1.</td>
<td>15</td>
<td>Nonius</td>
<td>53</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>10</td>
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<td>57</td>
<td>9</td>
</tr>
<tr>
<td>3.</td>
<td>5</td>
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<td>53</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>4.</td>
<td>9</td>
<td>Ardenez</td>
<td>53</td>
<td>52</td>
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</tr>
<tr>
<td>5.</td>
<td>12</td>
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<td>64</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>6.</td>
<td>16</td>
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</tr>
<tr>
<td>7.</td>
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<td>55</td>
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</tr>
<tr>
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<td>17</td>
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<td>62</td>
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<tr>
<td>11.</td>
<td>4</td>
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<tr>
<td>16.</td>
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<td>Ardenez</td>
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</tr>
<tr>
<td>18.</td>
<td>4</td>
<td>Ardenez</td>
<td>64</td>
<td>66</td>
<td>19</td>
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</tbody>
</table>

At the horses which presented laminitis, as a result of the removal of the lamellae and the dermal lamellae from the epidermal ones, according to Nathaniel A. White, the distance between the hoof wall and the dorsal edge is proximal
bigger than distal, this increasing the value of the third phalanx angle with the ground (13).

At non-lameness horses, the palmar angle is within the normal parameters with an average of 9.75 and a standard deviation of 0.5, while at the horses which presented lameness and were diagnosed with navicular syndrome, the palmar angle has an average of 7.1 and a standard deviation of 1.8, and in those diagnosed with laminitis the average is 16.16 and the standard deviation of 6.11.

Fig.1. Hoof – Horse without lameness  

Fig. 2. Hoof – horse with laminitis  

Fig.3. Hoof- horse with navicular syndrome
James K. Belknap reports that the rotation of the third phalanx inside the horn box leads to the decrease of the distance between the solear part of the third phalanx and the ground and the increase of the angle between the solear part of the third phalanx and the soil, known as palmar angle (3).

According to Andrea E. Floyd, the decrease of the palm angle below 10 ° occurs in the case of hoofs with short heels, long trimmed and with a high forehead, and in this case, the tension on the navicular bursa will be increased due to the deep digital flexor tendon with the occurrence of the navicular syndrome (5).

According to Joan Norton (8), at the horses with navicular syndrome, the lameness is visible in different circumstances, such as trapping in a circle, and while walking it can be noticed how the horse leans more on the forehead, avoiding the middle third of the frog and the heels, and more frequently thoracic limbs are affected. Ten horses out of those examined and diagnosed with navicular syndrome have presented lameness in certain circumstances, being characterized with grade 2 and 3 of lameness.

At horses diagnosed with laminitis, according to James K. Belknap, the degree of lameness will be a high one due to the possibility of affecting both the thoracic and pelvic limbs. If only the thoracic limbs are affected, the animal will stand with the thoracic limbs in the front and the support will be made on the pelvic limbs to reduce the weight on the thoracic limbs and the resistance caused by exercises is reduced and while standing, the animal has a characteristic position called "camped before and underneath him." When all 4 limbs are affected, the thoracic limbs are brought forward and the pelvic limbs are moved backwards, in which case the movement is difficult and the step is shortened while walking (3). Four of the examined horses showed laminitis with a high degree of lameness, respectively 4 and 5.

Conclusions

The radiographic exam is a precise method of diagnosis at the level of the hoof that offers the possibility of performing different types of measurements.

The radiographic image provides data on the position of the third phalanx and the distal sesamoid bone (navicular bone).

As a result of the measurement of the angles between the third phalanx and the adjacent structures within the hoof, the pathology of the hoof will be determined.

There are differences in the size of the angles and the degrees of lameness at the horses diagnosed with navicular syndrome versus those with laminitis.
Acknowledgements

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References

USE OF PLANT EXTRACTS WITH BIOCIDAL ACTIVITY IN ANIMAL SCIENCES RESEARCH AND VETERINARY MEDICINE

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Summary
Traditional medicine or folk medicine developed over many generations, starting in ancient time, was used to treat or prevent illness. Even today in many parts of the world, folk medicine is the only way to treat / alleviate disease. In the last few decades there has been a renewed interest in its knowledge and practices as a way to avoid some of the harmful side effects posed by the drug-based modern medicine. Plant extracts from a wide spectrum of plant species offer distinct advantages over the man-made chemical compounds such as very little side effects, if any, easy to formulate and administer, low cost of production and less stringent requirements from the regulatory agencies responsible for drug approval for human and animal use. Plant-derived biocidal compounds could be used on a large scale in industrial animal farms and poultry operations where overcrowding is a concern for animal health and well-being. A search of literature devoted to the subject shows a wealth of data on the biocidal activity of many plant species such as: Allium cepa – onion, Allium sativum – garlic, Cichorium intybus – cichorium, Cinnamomum ceylon – cinnamon, Curcuma longa – turmeric, Elettaria cardamomum – cardamom, Eugenia caryophyllata – clove, Foeniculum vulgare – fennel, Myristica fragrans – nutmeg, Nigella sativa – black cumin, Ocimum basilicum – basil, Origanum vulgare – oregano, Pimpinella anisum – anise, Sassafras albidum – sassafras, Trigonella foenum graecum L. – fenugreek, Zingiber officinale – ginger. Some of these plants have been used for some time in animal sciences experimental settings with good results.

Key words: biocide, plant extracts.

Over the last few decades we witnessed a renewed interest from physicians and consumers in some of the folk medicine approaches to treat / prevent a wide variety of medical conditions, both in humans and animals.

Since ancient times, humans looked to alleviate their ills or animal diseases to the plant kingdom. The history of herbalism goes back many centuries starting in ancient Mesopotamia, Egypt, India, China, Rome, and Greece. Historical evidences overlaps with that of food history – as many herbs and spices were used for food seasoning, as
medicinal remedies and anti-microbial agents. The imagine and written history of herbs dates back some 5000 years to the Sumarians, who created clay tablets with lists of hundreds of medicinal plants such as myrrh and opium. The study of plants continued in the ancient Egypt, India, China and contributed a great deal to our knowledge on how to use plants to our benefit. The Ayurvedic medicine is one such example. Closer to our times, in 50-70 AD Greece Pedanius Dioscorides published his "De Materia Medica" in which he listed over 1000 medicines from herbs, minerals and even animals (59, 62). His knowledge was widely utilized throughout the ancient period and Dioscorides remained the greatest expert on medicinal remedies until late Renaissance. In the 15th century Maior wrote his "Opera Medicinalia", which was a compilation of Greek, Indian, Persian, Arab and Babilionian texts (23).

The more recent history of herbalism in Europe goes back to the 16th and 17th centuries, when the text became available in English and other languages rather than Latin or Greek. The 18th and 19th centuries saw the beginning of scientific study of herbal extracts and the formalization of pharmacology as a field of science. Throughout time and science evolution, plants were seen as phyto-compounds used for the prevention, cure or help during the recovery from illness period. Thus, herbs were used in different forms, as fresh leaves or other parts of the plant (for example: wrapping wounds with leaves, chewing fresh or dried herbs), balms and lotions, teas, extracts (infusions, tinctures), and other forms (14, 37, 16, 51).

At times people used different forms of plant extraction such as: infusion; decoction; distillation of hydrosols; flower essences; tincturing by maceration, tincturing by percolation; wine infusion, vinegar infusion, glycerin infusion, oil infusion; ointments, salves and balms; lotions and creams, suppositories and boluses; herb jellies, syrups, honeys, oxymels and electuaries; herbal pills; bath and water therapy, poultices and fermentations (9, 21, 25, 27, 55).

The World Health Organization (WHO) defines traditional and complementary medicine as a compilation of knowledge, skills and also practices of beliefs, theories and experiences of various cultures in order to prevent, treat and recover after diseases (63).

Extracts of Allium cepa L. with biocidal activity

Allium cepa L. (common name onion), belongs to the Alliaceae family, and is used in worldwide cuisine, but also as curative remedy under different forms. Thus, the oil extract, obtained mainly by steam distillation contains a mixture of sulfured compounds with bioactive, and mostly biocidal activity. As the extract is a natural product, without any chemical added and having biocidal activity (inhibiting the growth of pathogenic bacteria)
with very low allergenic potential, and with antioxidant activity has also been used as anti-brewing agent in the food industry (22, 54, 57).

**Extracts of Allium sativum with biocidal activity**

The common name for *Allium sativum* Linn. is garlic, and is very much used in culinary field as a food spice. Garlic belongs to *Liliaceae* family and has medical benefits for gastrointestinal parasitic diseases, and for circulatory ailments. Garlic oil contain sulfur components that inhibit lipid peroxidation (acting as antioxidant), has antibacterial and antifungal activity, and is also used as anti-brewing agent – by inhibiting specific enzymes (34, 35).

**Extracts of Cichorium intybus with biocidal activity**

Common chicory (*Cichorium intybus*) is part of *Asteraceae* family, and the leaves are used in salads, and buds or roots (var. *C. sativum*) are used as coffee substitute. Chicory roots have volatile oils and have anti-parasitic action against intestinal parasites. Since old times chicory was used especially in Germany, for common ailments, gallstone treatment, gastro-enteritis. It contains inulin, which helps in body weight management, constipation as well as increases calcium absorption, and has anti-toxic action. It also has tonic effects on the body.

It has been recently shown that a mixture of aqueous leaf extract of *Cichorium intybus* and AgNO₃ yielded silver nanoparticles (AgNPs), which exhibited antibacterial activity (19). Aqueous and organic solvent (ethyl acetate and ethanol) seed extracts of *Cichorium intybus*, were tested and showed good antimicrobial activity. But, the aqueous seed extracts were more effective against *Staphylococcus aureus* whereas organic extracts were more potent against *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (47). Water and ethyl acetate crude extracts have very good activity against *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Fusarium solani*, while chloroform extracts had higher activity against *Fusarium solani* (45). Another study showed the antibacterial activity of *Cichorium intybus* aqueous, ethanol and ethyl acetate extracts. Thus, all extracts exhibited very good antibacterial activity on *Agrobacterium radiobacter* sp. *Tumefaciens*, *Erwinia carotovora*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, but ethyl acetate extracts were most active (42).

**Extracts of Cinnamomum ceylon with biocidal activity**

Cinnamon is well known all over the world for its beneficial medical properties, and also as a widely used spice. The most used cinnamon species are *Cinnamomum zeylanicum*, *Cinnamomum cassia* (known as Chinese cinnamon – low quality), *Cinnamomum osmophleum*, *Cinnamomum camphora*, and *Cinnamomum verum*, called Ceylon cinnamon tree.

*Cinnamomum cassia*, called Chinese cinnamon, is an evergreen tree originating in southern China, and widely cultivated there and elsewhere in southern and eastern Asia (India, Indonesia, Laos, Malaysia, Taiwan, Thailand, and Vietnam). Cinnamonom *Casya* is considered to have lower quality, being much cheaper to buy than *Cinnamomum Ceylon*. There is a very big difference between the two species as far as
the curative properties are concerned. *Cinnamomum cassia* has dark brown to red color, but the structure is compact and rougher than the Sri Lankan counterpart. *Cinnamomum Ceylon* originated from Sri Lanka and India and is considered the true cinnamon, being more expensive and having a higher quality than *C. cassia*. *C. cassia* is brown, and the structure consists of soft layers and tight sticks. *C. verum* (*C. Ceylon*) contains about 0.4-0.8% oil, tannins, catechins and proanthocyanidins (condensed tannins), resins, carbohydrates, mucilage, calcium oxalate, cinnzelanin and cinnzelanol – with insecticidal action, cinnamic aldehyde (60-80% in oil), cinnamyl-acetate, and also eugenol, linalool, limonene, and cumarine (lower concentration in *C. verum* compared to *C. cassia*).

The oil obtained from *C. cassia* has mainly cinnamic aldehyde (65.5% - 87%, depending on the origin). Pharmacological research demonstrated that extracts of cinnamon (*C. verum*) is efficient against *Candida albicans*, but the essential oil has antifungal and antibacterial activity, being used in human and veterinary medicine, and also in food storage technologies (20, 28).

Due to cinnamaldehyde, the extracts of dried stem bark exhibited intestinal antibacterial activity, while the methanol extract induced apoptotic cell death (53).

Other studies showed significant insulin-like activity of cinnamon extract – increasing the uptake of glucose and increasing glucose metabolism (4).

**Extracts of Curcuma longa with biocidal activity**

Turmeric or *Curcuma longa* is a rhizomatous perennial plant from Zingiberaceae family, like ginger. Turmeric was used from ancient times, in Ayurvedic medicine, due to curcuminoids compounds (like curcuma, demethoxy-curcumin, bisdemethoxy-curcuma), and also turmerone, atlantone, zingiberene and other volatile oil compounds, which exhibited antimicrobial activity. Besides its use in Indian traditional ceremonies and rituals, turmeric has anti-inflammatory activity.

An experimental study employed a turmeric extract embedded in a chitosan film that was tested for its antimicrobial effectiveness against *Salmonella* and *Staphylococcus aureus*. The film was obtained by a casting and cross-linking procedure with sodium sulfate, and the IR spectroscopy analysis indicated a possible interaction between the phenolic compounds in extract and the amino group in chitosan and that resulted in very good physico-chemical and antimicrobial properties for the turmeric extract (30). Gingivitis affects about 80% of world population and the classical treatment based on chlorhexidine has side effects (erosion of mucosa, teeth decoloration, and unpleasant taste). Turmeric extracts seem to be a very good substitute as recent tests demonstrated that turmeric has antibacterial, antiviral, antifungal, anti-inflammatory, and antioxidant activity (50).

Nanocurcumin water extract effects were compared to curcumin particle ethanol extracts on human oral microflora. Nanoparticles of curcumin proved to be more effective at disrupting bacterial metabolism and are more efficient against oral cavity microflora (24).
Extracts of *Elettaria cardamomum* with biocidal activity

Cardamom (*Elettaria cardamomum*) is a perennial red-like plant from Zingiberaceae family (like ginger). It contains about 2.8-6.2% volatile oils (mostly terpinyl acetate and 1,8-cineole), 10% proteins, 1-10% oil, up to 50% starch, but also important minerals (Mn, Fe). Cardamom also contains linalool, limonene, sabinene, linalyl acetate and other important terpenoid compounds (5, 31).

Extracts of *Eugenia caryophyllata* with biocidal activity

Clove or *Eugenia caryophyllata* is part of Myraceae family, and *Syzygium aromaticum* is the tree with aromatic flower buds. Ayurvedic and Chinese medicine used the oil extracted from cloves to relieve severe sore toothache. The oil is a natural antihelmintic product, and is used for different medical conditions such as: cramps and flatulence, tooth decay. Clove volatile oil is rich in eugenol >95%, eugenol acetyl, methyl salicylate, β-caryophyllene, vanillin, pinene – compounds with antiseptic (antibacterial, antifungal) activity (40). Research studies also indicated that *Eugenia caryophyllata* is not only bactericidal, but also effective as acaricides. Thus, essential oil of clove was tested for its efficacy against *Psoroptes cuniculi* (parasite responsible for sheep or cattle scaby) *in vivo* and *in vitro* on rabbits. The experimental group of infested rabbits topically treated with essential clove oil in a concentration of 2.5% was completely cured (18).

Clove essential oil was also tested as an antimicrobial natural preparation in chicken meat packaging. Thus, linear low-density polyethylene – LLDPE surface modified with chromic acid (CA) was treated with clove essential oil (CEO), and the material was used to package fresh chicken meat. This LLDPE-CA-CEO film demonstrated strong activity against *Salmonella typhimurium*, and *Listeria monocytogenes* in chicken meat packaging refrigerated for 21 days (39).

A nanoemulsion of blended cloves and cinnamon essential oils was tested as a biocidal agent against *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*. The nanoemulsion (particle size 8.69nm) was obtained using Tween 80 and ethanol, and then tested after either: 1) centrifugation at 10,000 rpm for 20 minutes; 2) stored at 60°C for one month; 3) heating at 80°C for 30 minutes. The nanoemulsion of clove and cinnamon was stable after all three methods of preparation and antimicrobial activity was high even at far lower concentrations, which demonstrates that this natural easy-made preparation is very useful as a food preservative, and also in animal and human health (58).

Extracts of *Foeniculum vulgare* with biocidal activity

Fennel (*Foeniculum vulgare*) is a perennial plant from Apiaceae / Umbelliferae family, with very aromatic flavor used in food cooking and medicine. The main bioactive components of fruits volatile oil are anethole (50-60%) and fenchone (15-20%) (36).

Biocidal activity of essential oil of leaves of fennel (*Foeniculum vulgare* Mill. ssp. Vulgare var. azoricum Thell) supplemented with anethole had good effect, and had a better activity against Gram-positive bacteria. Also, this essential oil was tested for antioxidant capacity and the FRAP test did not yield any positive result, but DPPH test showed a weak antioxidant action against free radicals (46).
Fennel seeds hydro-distillation oil contains 68.53% trans-anethole and 10.42% estragole. This oil preparation was tested for antibacterial activity (minimum inhibitory concentration and minimum bactericide concentration) in food products and resulted in good inhibitory activity against *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, and *Staphylococcus albus* (15).

**Extracts of Myristica fragrans with biocidal activity**

Nutmeg (*Myristica fragrans*), is used as seeds in food cooking and medicine. The seeds in the fruits of this evergreen tree have multiple medicinal applications. The extract of nutmeg seeds has antimicrobial and antioxidant activity, depending on the extraction solvent (acetone, ethanol, methanol, butanol, water). Acetone nutmeg seed extracts showed the highest antioxidant activity, and the highest antimicrobial activity against *Staphylococcus aureus* and *Aspergillus niger*. Possible components responsible for antioxidant and antibacterial activity are represented by sabinene (28.61%), β-pinene (10.26%), α-pinene (9.72%), myristicin (4.30%), isoeugenol (2.72%), carvacol (1.54%), eugenol (0.89%), and β-caryophellene (0.82%) – (26).

Nutmeg is also used in combination with other herbal spices (such as oregano) for its antibacterial activity. Thus, oil from nutmeg and oregano was used in the traditional Iranian barbeque chicken cooking to prevent contamination by *Escherichia coli*. Medium rare traditional Iranian barbeque chicken was deliberately contaminated with *E. coli*, and then treated with nutmeg and oregano oil and stored at 300 C, 800 C and 200 0 C. At specific times, the effect of oil was tested on the chicken barbeque and the results showed no antibacterial action against *Escherichia coli* despite the fact that the nutmeg and oregano oil had biocidal effect on *E. coli* in broth culture (48).

**Extracts of Nigella sativa with biocidal activity**

Black cumin (*Nigella sativa*) is an annual plant from *Ranunculaceae* family, originally from southwest Asia. It is also known as cumin or black cumin, black-caraway, panacea, nigella, fennel-flower, and its seed are used in medicine for prevention and treatment of various diseases, including different forms of cancer (1, 2, 3).

For many years the black seeds and black seeds oil was shown to have antimicrobial, anti-inflammatory, antioxidant, anti-hyperlipidemic, anti-diabetic, anti-asthmatic, and anticancer activity (33).

Gold nanoparticles containing essential oil extracted from *N. sativa* seeds (NsEO-AuNPs) were tested for antibacterial activity against *Staphylococcus aureus* and *Vibrio harveyi*. Gold nanoparticles – NsEO-AuNPs had inhibition action against *S. aureus* and *V. harveyi*, and also exhibited in vitro anti-lung cancer activity in A549 carcinoma cells (38).

Black cumin oil also showed biocidal activity against Gram-negative and Gram-positive bacteria such as *Penicillium citrinum*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and very good antioxidant activity (43).
Extracts of *Ocimum basilicum* and *Origanum vulgare*

with biocidal activity

Basilicum or basil (*Ocimum basilicum*) is an annual plant from *Lamiaceae* family, used in culinary, medicine, and religious (used for preparation of holy water) practice. Oregano (*Origanum vulgare*), like basil is an herbaceous perennial plant and belongs to the *Lamiaceae* family. Flavored leaves are commonly used as food spice, particularly in tomato sauces and pizzas together with a herbal oil and vinegar (60).

Single and mixtures of different herbs (basilica, caraway, oregano) extracts were tested against *Cladosporium cladosporioides*, *Aspergillus wentii*, *Eurotium herbariorum*, *Penicillium aurantiogriseum* and *Fusarium verticillioides*, which are often present in foodstuff. Binary extract mixtures showed the highest inhibitory effect on fungi compared to individual extracts (32).

Edible chitosan films with antimicrobial activity were tested to ensure food quality and safety. Active biomolecules of chitosan were treated with oregano essential oil and then tested. This bioactive antimicrobial film exhibited high activity against Gram-negative and Gram-positive bacteria (17).

Extracts of *Pimpinella anisum* with biocidal activity

Aniseed (*Pimpinella anisum*) is an annual plant from *Apiaceae / Umbeliferae* family, named commonly as “great burnet”. Nano emulsions of anise oil are more stable than crude emulsions and have antimicrobial activity against *Escherichia coli* and *Listeria monocytogenes* that often contaminate food products (52). Administration of anise seeds as substitute antibiotic growth promoter in broiler chicken showed that addition of 10g anise seed / kg live chicken is a good practice and it eliminates the need of using conventional antibiotics in the feed of broiler chicken (56).

Extracts of *Sassafras albidum* with biocidal activity

A combination of ethanol and aqueous extracts of *Sassafras albidum*, *Ehretia anacua*, *Melissa officinalis*, *Eysenhardtia texana*, *Melissa odorata*, was tested for its antibacterial activity against some bacteria and fungi (*Staphylococcus aureus*, *Pseudomonas aeruginosa*) (12). Heliotropin is a sassafras extract (known as piperonal or sassafras oil) used for a long time in perfumes and soaps, and also for antibacterial and antiviral activity. Heliotropin crystals are also used in illicit drug manufacturing such as Ecstasy and even if it has good antimicrobial activity, due to the possibility of being used as precursor for specific drugs, sassafras was banned in foodstuff preparation (13).

Extracts of *Trigonella foenum graecum L.* with biocidal activity

Ethanolic extract of roots from *Cicorium intybus*, leaves of *Ficus religiosa*, and leaves of *Trigonella foenum-graecum* exhibited good antibacterial activity and less anti-fungal activity (7).

Extracts of *Zingiber officinale* with biocidal activity

A recent study was carried out on six herbal (crude protein) extracts with antimicrobial activity. Extraction was performed in sodium phosphate – citrate buffer, and
sodium acetate buffer at different pHs. Very good activity was recorded for Allium ascolinicum seeds extract (pH 5.8) against Proteus vulgaris, Staphylococcus aureus, Escherichia coli, and Rumex vesicarius (pH 7.6). Ammi majus (pH 6.8), Cichorium intybus (pH 7.4), and Cucumis sativus (pH 7.8) showed very good activity against all the above bacterial strains. Also, different herbal extracts (sodium acetate buffer at pH 6.5) of Foeniculum vulgare and extracts of Cichorium intybus and Cichorium sativus had good inhibitory activity against these pathogens but not for all tested strains as some exhibited resistance. The good part is that in general, the extracts exhibited better antibacterial activity than chloramphenicol (25mcg), and this may give traditional medicine a competitive edge over chemical drug-based medicine that is still the method of choice for disease treatment in humans and animals (6, 8, 11).

Conclusions

Plant extracts are used in veterinary and human medicine and nutrition, being considered natural treatments. Specific plant extracts present biocidal activity and can be used in animal farms and poultry industry replacing with success some classical antibacterial drugs (biocidal drugs). The extracts can be used in different forms depending on the purpose and plant extraction method, such as: tinctures, infusion, ointments, salves, balms, lotions, creams, suppositories, syrups, herbal pills, bath therapy, fermentations. Also, these formulation are usually has low-cost production, easy to meet the regulatory requirements, and does not need special storage conditions.

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APPLICABILITY OF A DUPLEX END POINT PCR METHOD IN DETECTING SHEEP DAIRY PRODUCTS ADULTERATION BY COW MILK FRAUDULENT ADDITION

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Summary
The majority dairy products found on the local market are made from cow milk and the ones processed from sheep are considered regional specialties with characteristic organoleptic properties. In the industry, protection against fraudulent species substitution or undeclared admixture is important for several reasons, such as allergy adverse reactions toward some species specific milk proteins or trade and government regulations. The objective of the present study was to assess the purity of sheep dairy products labeled as “pure sheep” in the market. A duplex PCR method for the identification of cow’s milk constituent in sheep dairy products was validated. The limit of detection of cow’s milk in sheep’s dairy products is less than 1%. This research was conducted on a number of 15 different sorts of cheese collected from the local market. Total DNA was isolated and purified from 200 mg of raw material. All the tested samples were subjected to amplification with cow and sheep specific primers and majority of them were found to be mix with cow milk that was unlabeled or undeclared by producers. The validated technique is reliable, accurate and fast, also being suitable to be used in composition of dairy products testing in order to avoid unfair competition and to assure the correct labeling.

Key words: sheep cheese, adulteration, cow milk, detection, PCR

The high demand for milk and dairy products makes these aliments targets for potential adulteration with financial gains for unscrupulous producers (13). Milk-based products produced from sheep milk are located at the top of the list of healthy dairy products, earning the interest of consumers. Dairy products authenticity is of great demand in order to protect consumers from fraudulent products and health risk (10). But, these products are extremely vulnerable to adulteration with milk of lower added and nutritional value for profit. The most common adulterations of dairy products are the substitution of higher value milk by non-declared milk or the omission of a declared milk species. In addition, adulterant milks, such as bovine milk, have been accused to cause allergic reactions, especially to sensitive human groups. Thus, the detection of milk species
is important in cheese producing branch, especially those made from one pure species and with protected designation of origin, such as pure sheep (3).

In order to avoid the possible fraudulent substitution of sheep milk with cow’s milk, it is necessary to develop analytical methods that are able to detect frauds and protect the consumers from misleading labeling (5, 9, 14).

Different analytical approaches have been applied for composing species identification, most of them implying the detection of specific proteins, among these, immunological (7, 15, 16), electrophoretical (12), chromatographic (6), are the most used. The present European Community reference method for detection of cow milk and caseinate in cheeses made from sheep milk, goat milk, buffalo milk or mixtures of sheep, goat and buffalo milk is isoelectric focusing of γ-caseins after plasminolysis (4). Even if good results can be obtained with specified methods it has to be taken in consideration that the processed biological material are prone to denaturation which can alter the analysis result. In the last two decades more DNA based methods are being developed (1, 2, 3, 11). Those methods are based on PCR technique and use specie specific designed primers for targeting DNA sequences. In 2003, Bottero et al. developed three primer pairs that are specific for cow, goat and sheep targeting the 12 S rRNA mitochondrial DNA regions from their genome. After describing the specificity of those primers pairs, they were multiplexed in PCR amplification, this leading to a fast and accurate method for detection of the three species DNA in one single reaction and later, also a relative quantification of DNA being possible (8, 11).

Considering this framework the present paper describes a validation of a duplex PCR, developed in order to detect two component species: cow and sheep and also the applicability of this method in a screening study of 15 cheese samples collected from the autochthon market.

Materials and methods

The biological material consisted of internal prepared reference material used as positive control 100% cow and 100% sheep DNA (own work, procedure not described here) and 15 samples of different sorts of sheep cheese (Table 1).

The primers pairs, proposed by Bottero et al. (3) were used in this study. The primers were developed for the two species, targeting a mitochondrial DNA sequence - 12s rARN, generating amplicons of different lengths (Tabel 2). The primers were synthetized by Eurogentec (Belgium).

The DNA was extracted from 200 mg of raw material using Wizard® Magnetic DNA Purification System for Food (Promega, USA) commercial kit, following the producer’s instructions. Beside, an extraction blank control (EB) was done.
Table 1

<table>
<thead>
<tr>
<th>Nr.crt</th>
<th>Biological sample</th>
<th>Label composition</th>
<th>Provenience/type</th>
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<tbody>
<tr>
<td>1</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>Hypermarket/commercial product</td>
</tr>
<tr>
<td>2</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>Hypermarket/commercial product</td>
</tr>
<tr>
<td>3</td>
<td>Maturated cheese</td>
<td>sheep/cow</td>
<td>Hypermarket/commercial product</td>
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<tr>
<td>4</td>
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<td>cow/sheep</td>
<td>Hypermarket/commercial product</td>
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<tr>
<td>5</td>
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<td>sheep/goat</td>
<td>Hypermarket/commercial product</td>
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<td>sheep</td>
<td>Hypermarket/commercial product</td>
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<td>7</td>
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<td>sheep/water buffalo</td>
<td>Hypermarket/commercial product</td>
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<td>8</td>
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<td>sheep/cow</td>
<td>Hypermarket/commercial product</td>
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<td>sheep</td>
<td>local market, Timis county /traditional product</td>
</tr>
<tr>
<td>10</td>
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<td>sheep/goat</td>
<td>local market, Timis county /traditional product</td>
</tr>
<tr>
<td>11</td>
<td>Raw cheese</td>
<td>sheep</td>
<td>local market, Timis county /traditional product</td>
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<tr>
<td>12</td>
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<tr>
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<tr>
<td>15</td>
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<td>sheep</td>
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Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Primers sequence Forward/Reverse</th>
<th>Amplicons length /bp</th>
</tr>
</thead>
<tbody>
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<td>5’ GTACTAGCAACACGCTTA 3’ 5’GCTTGATTCTCTTGGTGTAGAG 3’</td>
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<tr>
<td>Ovies aries</td>
<td>5’ ATATCAACCACACGAGAGGAGAC 3’ 5’ TAAAAGGTGGGAGAT 3’</td>
<td>172</td>
</tr>
</tbody>
</table>

The quality and quantity of extracted DNA was assessed by spectrophotometry method (NanoDrop 8000 Spectrophotometer - ThermoScientific). DNA was evaluated directly in aqueous diluted solution, measuring the optical density (OD) in ultraviolet light. The concentration of nucleic acids was determined by measuring at 260 nm against a blank. The quality of extracted DNA was evaluated by A$_{260}$/A$_{280}$ ratio. The purity of extracted DNA was evaluated by A$_{260}$/A$_{230}$ ratio.

The enzymatic reactions were run in a final volume of 25 µl using KAPA 2G™ Robust HotStart ReadyMix (KapaBiosystems) according to producer instructions, 20 pmol of primers and 100 ng/µl DNA template on a Mastercycler ProS (Eppendorf U.S.) thermocycler.

The PCR program consisted of an initial denaturing step for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 60 sec and extension at 72°C for 1 min, with a final step at 72°C for 5 min.

The resulting PCR products were migrated by electrophoresis on 2.5 % agarose gels in TAE buffer at a constant voltage of 100 V for 40 minutes.
The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England).

**Results and discussion**

The reference samples for the in house validation procedure were prepared from DNA solutions, 100 ng/µl, isolated from uncontaminated biological samples, mainly standard cheese prepared in our laboratory. The concentrations of the reference DNA samples are listed in Table 3. The mixtures were prepared in order to cover a large area of cow milk addition. However the smallest concentration in the final reference sample was 0.5 % of cow DNA, since it was considered that under this threshold it is hardly to suspect a fraudulent addition, but rather an involuntary contamination.

<table>
<thead>
<tr>
<th>Specie</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>50</td>
<td>75</td>
<td>85</td>
<td>95</td>
<td>99</td>
<td>99.5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Cow</td>
<td>50</td>
<td>25</td>
<td>15</td>
<td>5</td>
<td>1</td>
<td>0.5</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

In the first step of the study, total genomic DNA was isolated and purified from the acquired cheese samples. DNA of amplifiable quality and quantity was obtained and serial dilutions were prepared in the attempt to equalize the genes of interest copies number that may be present in the DNA samples. As expected, the quantity and quality of isolated DNA from processed samples was lower but still considered suitable for PCR analysis.

The in house validation of the described duplex method was carried on and the results were interpreted according to PCR product intensity as it can be visualized in agarose gel (Figure 1).

In the case of sheep DNA it can be noticed that the result of PCR does not depend of sample concentration, since all the values exceeded 50%, it can be concluded that using this pair of primers the efficiency of PCR detection is optimal. For the cow biological material the dilution were decreased constantly, but the detection is still possible even at a very low concentration (0.5%). However, a detection that will exceed this minimum threshold can hardly be considered adulteration but rather an unwilling contamination. The method was considered valid and accurate by analyzing the result obtained in the case of reaction controls. The positive controls were represented by DNA samples prepared from pure raw material, cheese of sheep and cow milk, and by PCR amplification it was proved that the primers pairs are highly specific, since no unspecific amplicons were detected. The negative controls: negative template control (corn flour) and non-template control (reagent control) were found to be free of PCR product, that being a compulsory condition when the method validation is desired.
After the proposed method was proved to be valid, the next step of the experiment, the screening of the collected samples was carried out. The DNA extracted and purified from the 15 cheese samples was subjected to duplex PCR analyses, along with the positive control which was represented by the reference sample prepared with 50 – 50 %, DNA of sheep and respectively, cow milk (Figure 2). Also, in this case two negative controls – the negative template (corn flour) and the reagent control were used. In each prepared reaction the same amount of DNA was used, namely 100 ng/ 25 µl final reaction volumes.

The proposed method is a qualitative one, so the results are interpreted only by presence/absence measure. Presence of a 256 bp amplicon was considered positive for cow milk, whereas the presence of a 172 bp amplicon was considered positive for sheep milk detection (Figure 2). The result of the screening is listed in Table 4.

According to our determination, only samples 1 and 3 composition was in concordance with the label description of the product. Those samples were acquired from the supermarket and are commercial products.
Fig. 2. PCR analysis for cow milk adulteration detection in cheese samples. Lane 1 – 15: the cheese samples used in this study (Table 1); lane 16, negative control – corn flour DNA; lane 17, positive control, Sheep/cow 50 - 50 % reference sample; lane 18, control reagent; M - molecular weight marker: molecular weight marker: PCR marker, (Promega, USA)

<table>
<thead>
<tr>
<th>Nr.crt</th>
<th>Biological sample</th>
<th>Label composition</th>
<th>PCR detection result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>sheep</td>
</tr>
<tr>
<td>2</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>3</td>
<td>Maturated cheese</td>
<td>sheep/cow</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>4</td>
<td>Maturated cheese</td>
<td>cow/sheep</td>
<td>cow</td>
</tr>
<tr>
<td>5</td>
<td>Maturated cheese</td>
<td>sheep/goat</td>
<td>sheep</td>
</tr>
<tr>
<td>6</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>7</td>
<td>Raw cheese</td>
<td>sheep/water buffalo</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>8</td>
<td>Raw cheese</td>
<td>sheep/cow</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>9</td>
<td>Raw cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>10</td>
<td>Raw cheese</td>
<td>sheep/goat</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>11</td>
<td>Raw cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>12</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>13</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>14</td>
<td>Raw cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
<tr>
<td>15</td>
<td>Maturated cheese</td>
<td>sheep</td>
<td>sheep/cow</td>
</tr>
</tbody>
</table>

Conclusions

The duplex PCR method proposed in this study can be considered as a further improvement of a PCR based assay for the control of dairy products. The specificity of the proposed primers pairs was proven by the validation experiment, along with the assay sensitivity and accuracy. The obtained results pointed out the need for authorized controls on the market, especially in the case of local producers that are using the adulteration as a common practice.
The test could be useful in the control of dairy products, to verify the origin of the raw materials, especially in products submitted to denaturing technologies, for which other methods cannot be applied.

Acknowledgements

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References

THERMAL IMAGING OF THE DOGS WITH CRANIAL CRUCIATE LIGAMENTS RUPTURES

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Summary
This paper work reviews the main clinical and imaging diagnostic methods of cranial cruciate ligament rupture in dogs and shows the results of using Flir E4 thermal imaging camera and Flir tools 2017 software analysis. Compared to the achievements of a correct cranial cruciate ligament ruptures diagnosis in dogs of 75-85% reported by Infernuso et al, 2010 using Med 2000 IRIS type thermographic camera, Meditherm Inc with intranet service for image interpretation and analysis with Automated Computer Vision software and Image processing-Algorithm Test and Analysis Tool (C VIP-ATAT) data obtained by us with Flir E-40 thermal imaging camera and Flir tools - 2017 analysis software did not allow the recognition of one pattern for differentiation between dogs with healthy cranial cruciate ligament (CCL) and with cranial cruciate ligament rupture (CCLR) as well as with knee osteoarthritis (OA). Thermal imaging camera and tested software allows differentiation of dogs without knee pathology by those with injured knees, without revealing significant differences among different pathological entities (CCLR versus OA).

Key words: dog, cranial cruciate ligament injury, thermal imaging diagnosis

Rupture of the cranial cruciate ligament (CCLR) is the most common cause of lameness in dogs (9, 18, 22, 27). Trauma accounts for a minority of CCL ruptures in dogs, whereas progressive degeneration of the ligament has been attributed to a variety of factors that may be broadly classified as genetic, conformational, environmental, immune-mediated, and inflammatory (9, 16).

An accurate diagnosis of cranial cruciate ligament (CCL) injuries is obtained through several clinical and imaging evaluation techniques. Clinical variables that were assessed included: lameness, palpable joint instability, articular crepitus, and joint swelling. Radiographic features that were evaluated included soft-tissue swelling/joint effusion, subchondral sclerosis, periarticular osteophyte and enthesiophyte formation, remodeling of femoral and tibial condyles, and resorptive changes in the intercondyloid fossa (39). Clinical diagnostic tests include drawer test and tibial compression test (19). A cranial drawer sign was detected in 13 of the 25 dogs; in 9, the cranial drawer sign was evident only when the stifle was positioned in flexion and 12 had no detectable cranial drawer sign in response to manipulation of the involved stifle (32). The comparison of the sensitivity and specificity of the drawer test (DT) alone and in combination with the tibial compression test (TCT) show that DT had a poor...
sensitivity for correctly identifying CCL (69%) and combining DT and TCT did not increase sensitivity or specificity values (26).

Carobbii and Ness, 2009 (8) in their preliminary study evaluated tests used to diagnose canine cranial cruciate ligament failure in conscious dogs, using cranial drawer, tibial compression, patellar tendon palpation and palpation of the medial aspect of the joint, in dogs under general anaesthesia, in which cranial drawer and tibial compression tests were repeated, and have achieved a lateral stifle radiograph. The obtained results show that the sensitivity of the cranial drawer and tibial compression tests was surprisingly low when performed on conscious patients but significantly better when performed under anaesthesia. Similarly, palpation of the medial aspect of the stifle joint cannot be considered a reliable indicator of cranial cruciate ligament injury. Patellar palpation and radiographic assessment showed excellent sensitivity, specificity, positive predictive value and negative predictive value. The sensitivity of the radiographic tibial compression test was 97 per cent, compared with 86 per cent for the cranial drawer test (10). Palpation of cranialolateral stifle laxity has become pathognomonic for CCL rupture; however, chronic periarticular fibrosis, a partial CCL rupture, and a tense patient, may make evaluation of instability of the stifle difficult (21). Radiographic measurement of the posterior-inferior tibial slope (PITS) indicates that an increased PITS is associated with CCL rupture (7). The detection of deep stifle ligaments (cranial cruciate ligament and caudal cruciate ligament) was extremely difficult to perform using ultrasonography (15). For cranial cruciate ligament rupture, the sensitivity for ultrasonographic diagnosis was 15.4% (1).

The global sensitivity of magnetic resonance imaging for the diagnosis of a meniscal tear consecutive to cranial cruciate ligament injuries was 100% and the specificity was 94% (sensitivity and specificity were 0.93) (4, 5) unlike of low-field magnetic resonance imaging (lfMRI) which was of low diagnostic accuracy in detecting meniscal tears (sensitivity and specificity were 0.64), when compared with arthroscopy (6) but low-field magnetic resonance imaging arthrography allows easy detection of CCL morphology (3, 28). The use of stress (tibial compression test and a cranial drawer test) MRI for evaluation of stifle joints of dogs with an intact or deficient cranial cruciate ligament shows that both stress techniques were measurable by use of MRI (36). Computed tomographic arthrography (CT arthrography) allows correctly identification of the solitary caudal cruciate ligament tear, having a specificity ranged from 89.3% to 100% (31, 38) The detection of injuries to the medial meniscus in dogs with naturally occurring cranial cruciate ligament insufficiency by computed tomography arthrography images was 57 to 64 per cent sensitive and 71 to 100 per cent specific for diagnosing medial meniscal injuries (35). Ralphs and Whitney - 2002 (29) through arthroscopic investigation on 100 dogs found that there is a strong association between CCL injury and lateral and medial meniscal injuries in dogs.

The thermographic symmetry has been evaluated in the limbs of healthy dogs, and no significant difference between the left and right legs was found, suggesting that thermal abnormalities between the sides could be used as a
diagnostic tool for dogs (24). The capability of thermography for differentiation between normal stifles and those with cranial cruciate ligament rupture in dogs has been reported (20). Thermography was successful in differentiating naturally occurring CCL-deficient stifles in dogs, with a success rate of 75–85% (20).

The purpose of this study was to describe a thermographic imaging protocol, with Flir model E4, equipment which is available within the surgery laboratory from Horia Cernescu Complex of research laboratories of the Faculty of Veterinary Medicine Timișoara for diagnosis of the cranial cruciate ligament (CCL) rupture in dogs.

Materials and methods

Inclusion criteria were clinical cases of dogs weighing over 20 kg body weight with unilateral CCL tear based on physical examination findings and confirmed by radiographic imaging (fluoroscopic), and arthroscopy or arthrotomy – table 1. Control group (dogs with intact CCL) was composed of 7 dogs, of which 4 were healthy and 3 were suffering from knee osteoarthritis (OA) – table 1.

Flir E-40 type thermographic camera with a 80x60 resolution, thermal sensitivity of 0.15°C, field of view 45°x34°A and uncooled microbolometer was used. Animal acclimatization with indoor examination space (at room temperature 20–23°C) was 30 minutes. Thermographic imaging of cranial (Cr) and lateral (L) views of the both stifles were obtained after the protocol presented by Inferusso-2010 (20). The region of the stifle was defined as the cranial aspect of the patella to the caudal aspect of the gastrocnemius muscle and the distal third of the femur to the proximal third of the tibia. Two sets of thermographic images for each dog were realised from a distance of 0.6-1 m. All images were obtained in a standing position after clipping the hair coat (with 24-48 hours before the shooting).

Software program Flir tools – 2017 freeware, was used to save, analyze, and review image data. The program calculated the mean, maximum, and minimum temperatures of each image for each views Cr and L. Results are reported as the adjusted mean±std. The relationship between the differences between superficial temperatures in the three groups considered for the study was assessed with t-Student models for each group.

After thermographic evaluation was completed, CCL deficient dogs were anesthetized and fluoroscopic, arthroscopic investigations or /and arthrotomy diagnosed and surgical treatments (fabellar-tibial suture was placed using monofilament nylon (14) or lateral suture stabilisation with bone anchors F2-T2 (17, 30) was performed.
Dogs enrolled in the study

<table>
<thead>
<tr>
<th>Crt no</th>
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<th>Breed</th>
<th>Age year</th>
<th>Gender</th>
<th>Weight kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCLR</td>
<td>half breed</td>
<td>8</td>
<td>F</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Caucasian Shepherd</td>
<td>3</td>
<td>M</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Husky</td>
<td>3</td>
<td>F</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>Cane corso</td>
<td>4</td>
<td>M</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Rottweiler</td>
<td>1.5</td>
<td>F</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>half breed</td>
<td>1</td>
<td>M</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>English Bulldog</td>
<td>1</td>
<td>F</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>half breed</td>
<td>4</td>
<td>M</td>
<td>28</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>Bulldog</td>
<td>5</td>
<td>F</td>
<td>20</td>
</tr>
<tr>
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<td>Control group</td>
<td>half breed</td>
<td>7</td>
<td>M</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>intact CCL</td>
<td>Rottweiler</td>
<td>3</td>
<td>F</td>
<td>55</td>
</tr>
<tr>
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<td></td>
<td>Rottweiler</td>
<td>4</td>
<td>F</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>half breed</td>
<td>4</td>
<td>M</td>
<td>24</td>
</tr>
<tr>
<td>1</td>
<td>Control group</td>
<td>half breed</td>
<td>8</td>
<td>M</td>
<td>33</td>
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<td>23</td>
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<tr>
<td>3</td>
<td></td>
<td>half breed</td>
<td>9</td>
<td>M</td>
<td>24</td>
</tr>
</tbody>
</table>

Mean±std

4.59±2.64

33.56±12.38

Results and discussions

The results obtained are shown in table 2 and figure 1 showing significant temperature differences between normal and CCL-deficient stifles (p=0.0008 for cranial view and p=4.19×10⁻⁵ for lateral view) and no significant differences between CCLR and OA stifles (p=0.97 for cranial view and p=0.94 for lateral view).

Analysis of images obtained allowed the detection of deficient stifle (CCLR and OA) which exhibited uniform in pattern distribution yellow to orange to red on the cranial views and yellow to orange in lateral views /compared with normal stifle joint uniform in pattern distribution blue to green in cranial image and blue to yellow in lateral image - fig. 1.

Compared to the achievements of a correct cranial cruciate ligament ruptures diagnosis in dogs of 75-85% reported by Infernuso, 2010 (20) using Med 2000 IRIS type thermographic camera, Meditherm Inc with intranet service for image interpretation and analysis with Automated Computer Vision software and Image processing-Algorithm Test and Analysis Tool (CVIP-ATAT) data obtained by us with Flir E-40 thermal imaging camera and Flir tools - 2017 analysis software did not allow the recognition of one pattern for differentiation between dogs with healthy CCL versus CCL rupture as well as with knee OA.
This fact is probably due, on one hand, to thermal sensitivity/NETD lower than $<$0.15°C (0.27°F) / $<$150 mK and to low resolution (80x60) fact explained by the low cost of 995.00 $ (13) compared with 34,850.00 $ for Med2000 IRIS Camera (34) and on the other hand, to differences between analysis software used. The camera resolution used in other studies in small-sized animals has often been 320 x 240 pixels (20, 24, 25). Three different resolution cameras (FLIR i5 80 x 80 pixels, FLIR b60 180 x180 pixels and FLIR T425 320 x 240 pixels) were compared using between-interpreter variation and between-thermographer variation as indicators for the comparison by Vainionpää, 2004 (37) which finds that with camera i5, the variation between thermographers was notably higher, almost 0.4 (0.27–0.6) and in a low-resolution camera such as i5, small changes in the image cannot be interpreted and placed in perspective due to the lack of resolution.

The thermographic imaging has been found reliable and objective in measuring the sings of inflammation in the knee joint in humans (11), and it has also been used for diagnosing cranial cruciate ligament rupture in dogs with no clinical findings of stifte laxity (20).

**Surface temperature values recorded by thermograph**

<table>
<thead>
<tr>
<th>No</th>
<th>Group</th>
<th>Deficient limb</th>
<th>Normal limb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max °C</td>
<td>Min °C</td>
</tr>
<tr>
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<td>CCLR</td>
<td>34.7</td>
<td>32.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>35.6</td>
<td>33.4</td>
</tr>
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<td>34.9</td>
<td>31.1</td>
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<td></td>
<td>32.5</td>
<td>32.3</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>34.0</td>
<td>30.8</td>
</tr>
<tr>
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<td>33.5</td>
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<th>Deficient limb</th>
<th>Normal limb</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Max °C</td>
<td>Min °C</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>31.4</td>
<td>34.9</td>
</tr>
<tr>
<td>2</td>
<td>OA</td>
<td>32.6</td>
<td>27.3</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>34.3</td>
<td>28.7</td>
</tr>
</tbody>
</table>

**Legend** – undetermined values

![Table 2](https://example.com/table2.png)
The distance from which the images have been carried in our study fits in the data from specialty literature - 1.5-4.6 m (24).

Acclimatization of the subject to the surrounding environment is essential in order to produce standardized, good-quality images (37). Acclimatization mean time used by us (30 minutes) is in accord with data and findings from the specialty literature which shows that clipping is not necessary for successful thermographic evaluation of the canine stifle (20, 24).

The development of new software and techniques for image analysis in medical thermographic imaging is an ongoing process (37). In some studies, more advanced purchasable software (2, 20, 23, 33, 40) has been used.

The limitations of our study are dictated both by the fact that there is little data on thermography used for diagnosis in small animals, and the fact that the FLIR E-4 thermographic camera is net inferior to other cameras used in medical studies (37).

**Conclusions**

Flir E-40 thermal imaging camera and Flir tools - 2017 analysis software allows only the differentiation of dogs without knee pathology from those with injured knee without significant differences between different pathological entities (CCLR versus OA).

**Acknowledgements**

This study was realised using the support and infrastructure project "Dezvoltarea infrastructurii de cercetare, educație și servicii în domeniile medicinii veterinare și tehnologiilor inovative pentru RO 05", cod SMIS-CSNR 2669.
References


COMPUTED TOMOGRAPHY AND RADIOGRAPHIC TECHNIQUE IN EVALUATION OF THE SPINE IN DOGS

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e-mail: robert.purdoiu@usamvcluj.ro

Summary

Accurate evaluation of the spine and identification of the lesions is an important step for surgical planning or treatment protocol. Until a few years ago, the myelography has been the standard diagnostic tool for assessing of spinal lesions. Even if the accuracy of myelography range between 40%-97% (1), myelography present a series of risks like seizures, apnea, cardiac arrhythmia, different cases of inflammation and even death (2-4).

The anatomical structures and the consistence of the tissues make the evaluation of the spine a challenging exam.

The biological material was represented by 10 dogs with the age ranging from 2 year to 9 year. The biological material was split in two group, the first group consist of five dogs that undergo evaluation trough radiography with or without contrast, and the second group form by five dogs undergo radiographic evaluation followed by CT evaluation.

Radiographic evaluation is a cheap and quick method to evaluate the osseous part of the vertebral column, but have limitation in high lighting the compressive processes that could appear in the medullary canal.

The native CT and contrast CT of the spine present certain advantages compared with the radiographic technique because allow the clinician to perform the postprocessing of the images and obtain more information regarding the vertebrae’s and the vertebral canal.

Key words: dog, vertebral column, spinal cord, Computed Tomography, radiography

Development of imaging technique open a new era in evaluation of the vertebral spine in dogs. Radiography is the easiest and common way to evaluate the vertebral spine in animals (1, 5), but the technique present certain limitation because of superimposing of the structures, the 2D image obtained is a replica of a 3-dimensional object. Radiographic evaluation of the spine technique was enhanced once the myelography procedure was introduces (6, 7, 8), but that method present also a risk of exacerbating the compression or the neurological signs (5, 9, 10, 11, 12). Using up to date CT scanner is possible to obtain a multiplanar reconstruction of the spine that help for a better evaluation of the spine canal and spinal cord. The possibility to adjust the HU window allow evaluation
both of the bone structure as well as the soft tissue. That represent a plus in
diagnostic of intervertebral disk disease of other degenerative spine diseases.

Material and methods

The biological material was represented by 10 dogs with the age ranging
from 2 year to 9 year. The biological material was split in two group, the first group
consist of five dogs that undergo evaluation trough radiography with or without
contrast, and the second group form by five dogs undergo radiographic evaluation
followed by CT evaluation. Both on radiography and CT examination the patients
were sedated using Xylazine (Bayer,USA) 2% 4 mg / animal i.m., Midazolam
(Bayer, USA) 5% i.v., Propofol (Pfizer, USA) 5 mg / animal by intravenous injection.
For the radiographic evaluation, the dogs were putted in latero-lateral decubitus
and a latero-lateral exposure of the spine was performed. For the CT evaluation,
the patient was put in dorso-ventral decubitus.

Image acquisition. For the radiographic examination, a TEMCO-GRX X-Ray
device with Xmaru 1717SGC/SCC Flat Panel. The DX modality acquisition was
performed using a 0.127/0.127 pixel spacing and fixed grid, the images were
visualized on Xmaru VetView acquisition software. Post processing of the images
were done using RadiAnt DICOM viewer and Weasis DICOM viewer, both of them
being free software's.

The CT examination was done on a 16-slice scanner (Siemens Somatom
Scope – Siemens Healthineers Global USA). The helical CT scanning was
performed on the vertebral column, using 130 kV, 150 mA and an exposure time
per rotation of 1 s and 0.85 pitch ratio. The scanning time for the spine was
68.12±3.4 s depending of the scanned area or the length of the scan, mean
CDTIvol being of 5.5 mGy.

The obtained images have a 512x512 matrix, using a soft tissue kernel. The
images were processed on Syngo Somaris/5 and on free software RadiAnt DICOM
viewer and Weasis DICOM viewer.

Results and discussions

The myelography is a useful tool in evaluation of the vertebral spine, but
present a high challenge in case of thoraco-lumbar or lumbo-sacral injection of
contrast media (1). Although the myelography is relevant in case of intervertebral
disk disease (IVDD) or hematoma (13) (Fig. 1), another downside of the
myelography evaluation is represented by the risk of increasing the intravertebral
pressure and compression or by inflicting neurological signs (2, 15).
Fig. 1. Myelography of the cervical spine, subdural hematoma (white arrow).

The radiography is useful in case of degenerative disease like spondylolisthesis deformans (SD) in which the new bone proliferation, calcification pons that form underneath the vertebra, give a very good radiographic contrast. Contrast agents used in myelography are very useful in diagnostics of nonmineralized intervertebral disk protrusion (16). In radiography, a better evaluation of the spine is obtained by performing two exposure, one in latero-lateral decubitus and the second one in dorso-ventral decubitus, the slightest angulation of the spine will alter the result.

Computer Tomographic evaluation present the advantage that allow the multiplanar reconstruction (MPR) of the scanned area without repositioning the patient (16) (Fig. 2).

Fig. 2. MPR rendering of the spine in vertebral canal stenosis, due to calcification of the ventral spinal ligament
Recommendation for CT evaluation of the spine is to be perform on only the interested region if it is possible, to avoid malposition of the body and high dosage of radiation from CT. Houndfield Windows help reconstruction and evaluation of the soft and bone tissue, that make the CT a useful method for qualitative and quantitative evaluation of the spine (Fig. 3).

Fig. 3. Qualitative and quantitative evaluation of the spine

Computed Tomography is helpful in evaluation of the changes that can affect the bone, but also changes in the soft tissue that can narrow the vertebral canal or displace the spinal cord (Fig. 4, Fig. 5, Fig. 6).
Fig. 4. Displacement of the spinal cord due to fat tissue reaction and narrowing of the nerve root opening

Fig. 5. Reaction of the dural sac and loss of density due to loss of fat tissue

Fig. 6. Calcification of the ventral spinal ligament
Conclusion

The native CT and contrast CT of the spine present certain advantages compared with the radiographic technique because allow the clinician to perform the postprocessing of the images and obtain more information regarding the vertebrae’s and the vertebral canal.

References


EVALUATION OF BIOACTIVE POTENTIAL OF COLLAGEN BASED BIOMATERIALS

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Summary

The aim of our study was to compare the bioactive effect of two types of collagen based biomaterials (Matri™ Bone Ortho, ChondroGide®) in combination with adipose tissue derived sheep stem cells. After isolation the cells were seeded on the selected biomaterials at a density of 2×10³ and the cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C. Cell adhesion, viability, cell proliferation, alkaline phosphatase (ALP) activity were evaluated. Our results suggest that this biomaterials promoting the cells adhesion, proliferation, without altering viability and morphological characteristics of the cells. We have also demonstrated the bioactive potential of collagen based biomaterials and might lead to an upgrading in the current clinical treatment of bone defects for veterinary medicine.

Key words: stem cells, sheep, biomaterials, bioactive, regenerative therapy.

Regenerative medicine is a new rapidly growing field focused on tissues engineering as well as to normalize congenital defects (7). Regenerative therapy of bone is currently one of the most important and challenging tissue engineering approaches in regenerative medicine (3). The goal of tissue engineering involves obtaining a three-dimensional (3D) biocompatible support that can be transplanted into damaged tissue (5). Collagen is the most widely distributed class of proteins in the human and animal body, are a key structural element of vertebrate evolution (9). The collagen is one the most studied biomolecules of the extracellular matrix (ECM). Collagen-based biomaterials for tissue engineering applications have been intensively investigated in past decades (9). Collagen scaffolds have been used to culture a wide variety of stem cells for different tissue engineering applications. Cocultured with primate embryonic stem cells aggregated in embryoid bodies can differentiate into neural and endothelial phenotypes (4, 6, 8, 12). Baharvand et al. (2006) a similar differentiation ability were demonstrated, they were used 3D collagen scaffolds in order to generate hepatocytes from human embryonic stem cells. In other studies a combination of collagen with fibronectin were used (2). Our study investigates the in vitro cytotoxicity and bioactive property of collagen based biomaterials.
Materials and methods

Mesenchymal stem cells were isolated from sheep adipose tissue. The study was approved by the Ethical Board of the Iuliu Hatieganu University (237/19.06.2014). The tissue fragments were digested using 0.1% collagenase IV (Gibco). Cells suspension and the resulted explants were cultured in Dulbecco's modified Eagle's medium /F-12 (Sigma-Aldrich) supplemented with 10% fetal calf serum (Gibco), 5% horse serum (Gibco), 1% antibiotic-antimycotic (Gibco) and 1% non essential aminoacids (NEA), (Sigma-Aldrich). The cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C. After 72h of culture, the medium was replaced and tissue pieces were removed.

The isolated cells were characterized after six passages using monoclonal antibodies against, CD44 and CD34 (BD Biosciences, San Jose, CA, USA). The prepared samples were analysed using a FACS Canto II flow cytometer and the DIVA software (BD Biosciences, San Jose, CA, USA). Data from 10,000 events were recorded. After immunophenotyping the cells were seeded on two types of collagen based biomaterials (Matri™ Bone Ortho, ChondroGide®). The initial cell seeding density was 3x10³ for 10 mm × 5 mm biomaterial. After 24 h the cytocompatible potential of collagen based biomaterials was evaluated using live/dead staining kit (Sigma-Aldrich). Samples were assessed using fluorescence microscopy (Nikon). Cell adhesion and cell proliferation were assessed with the Alamar blue test. The cultures were incubated for 1 hour at 37°C, in the dark. The supernatant was transferred to another 12-well plate and fluorescence intensity was measured using a BioTek Synergy 2 plate reader (540 nm). Bioactive property of biomaterials (Matri™ Bone Ortho, ChondroGide®) were evaluated after 7 day of seeding, alkaline phosphatase (ALP) activity were assessed in cell lysates and culture medium using fluorometric Alkaline Phosphatase kit (Sigma-Aldrich). Statistical analysis was performed using GraphPad Prism 6 software (Manual Graph Pad Prism 5.0, GraphPad Software). A value of \( p < 0.05 \) was considered statistically significant. Data were reported as the mean ± SD.

Results and discussions

Mesenchymal stem cells were isolated from sheep adipose tissue using enzymatic treatment in combination with explants culture procedure. After confluence, the flow cytometry analyses confirmed positive expressions of mesenchymal cell-associated marker (CD44) and negative expressions of hematopoietic marker (CD34) (fig 1). The cells were observed throughout the entire culture period.
In order to evaluate the proliferation level of cells in collagen based biomaterial we used the characterized cells culture. After 24 h the adherence level and biocompatible potential of sheep cells to the biomaterials was evaluated using live/dead staining kit (fig 2). After 24 h the cells formed small aggregates on biomaterials, these microaggregates were counted after 48h.

No significant variants were identified between the two types of biomaterials in the degree of attachment and proliferation; cellular proliferation and cells aggregation was supported equally. The number of mesenchymal stem cells on the surface after 24 h of growth was essentially similar on all the biomaterials evaluated.

The number of aggregates on Matri™ Bone Ortho was 71.66± 4.49 and 70.33±4.68 on ChondroGide®. On Matri™ Bone Ortho the cells aggregates
observed were smaller (less than 30 cells) compared with ChondroGide®. Alkaline phosphatase activities were measured after 7 days of cell lysate, using a colorimetric ALP detection kit (fig.4). The results of the two evaluations were similar; no statistically significant differences were recorded. Our observations are in agreement with certain data from the literature.

In order to fabricate a scaffold, ideal biomaterials have to possess some specific properties. Of these, the most important features are biocompatibility, biodegradability and also appropriate mechanical properties (11). For orthopedic applications an ideal scaffold should also be an osteoconductive material, with the ability to that stimulates bone cells proliferation and differentiation on its surface (3).

The biomaterial should also be suitable for sterilization to prevent infections and be interconnected and demonstrate controlled porosity (10, 3). Furthermore, it is also important to integrate easily into the tissue should be able to undergo efficient resorbable at the same time that the bone regenerates, without any immunogenicity or cytotoxicity (11). Collagen-based biomaterials through their capabilities such as biocompatibility and low immunogenenicity represent ideal scaffolds for regenerative therapy (9).

Conclusions

Our results suggest that this biomaterials promoting the cells adhesion, proliferation, without altering viability and morphological characteristics of the cells.

We have also demonstrated the bioactive potential of collagen based biomaterials and might lead to an upgrading in the current clinical treatment of bone defects for veterinary medicine.
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