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ŞI MEDICINĂ VETERINARĂ A BANATULUI
TIMIŞOARA**

FACULTATEA DE MEDICINĂ VETERINARĂ

LUCRĂRI ŞTIINŢIFICE

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EARLY RETINAL DEGENERATION IN A *MINUET* CAT: A CASE REPORT

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Summary

The purpose of this paper is to describe the clinical and electroretinographic findings in a case of early retinal degeneration in a Minuet cat. The visual deficits were first noticed by the owner at 4 months of age, but the cat was presented in our clinic with complete blindness at 7 months. The ophthalmic examination revealed mydriasis in ambiental light, negative menace response, normal intraocular pressure. Chromatic pupillary light responses were negative to the red light (480 nm) and positive to the blue light (630 nm). The fundus was examined by direct ophthalmoscopy and included the following changes: hyperreflectivity of the tapetum lucidum, severe attenuation of the retinal vessels, optic disc atrophy. The recorded electroretinogram (Dog Diagnostic Protocol, HMsERG device) showed non-recordable a and b waves. The owner declined to perform genetic testing of the patient. Both patient's parents had normal vision, but were carriers (N/PRA-pd) of the AIPL1 gene variant (577C>T mutation), which suggests that this could be the gene involved in this early retinal degeneration. The Minuet cat has an obvious risk for progressive retinal atrophy, since Persian cats were used in the foundation of this recently developed breed but, to our knowledge this is the first report of early onset PRA in the Minuet cat in Romania.

Keywords: Minuet, cat, progressive retinal atrophy, electroretinography

Progressive retinal atrophy (PRA) is a collective term for a group of inherited retinal diseases that primarily affect the photoreceptors and causes blindness in animals.

Affected animals reveal initially night blindness due to degeneration of rod cells, then the disease progresses, affecting the cone cells leading to complete blindness.

PRA may be classified into two groups. One group is the early-onset, photoreceptor dysplasia, which affects photoreceptors before they are fully mature and rapidly leads to progressive retinal degeneration; while the other group is the late-onset photoreceptor degeneration, which occurs after normal retinal development, then followed by generalized photoreceptor degeneration (7, 14).

Four inherited forms of PRA are documented in domestic cats: an early-onset, dominantly-inherited rod–cone dysplasia (rdy), a late-onset recessively-inherited rod–cone degeneration (rdAc), both in Abyssinian cats; an early onset, recessively-inherited rod–cone dysplasia in Persian cats, and in Bengal cats, an autosomal recessive PRA (3, 4, 12, 14, 15).

The Minuet cat is a relatively new breed, founded by hybridization of a

Munchkin with a Persian cat. This article aims to present the clinical and electroretinographic findings in a case of early retinal degeneration in a Minuet cat.

Materials and methods

The 7 months old cat was presented to the Ophthalmology Department of the Faculty of Veterinary Medicine in Bucharest with complete blindness. The visual deficits were first noticed by the owner at 4 months of age. The ophthalmic examination revealed mydriasis in ambiental light (Fig. 1.), negative menace response and negative cotton ball, normal intraocular pressure. Chromatic pupillary light responses were negative to the red light (480 nm) and positive to the blue light (630 nm). The fundus was examined by direct ophthalmoscopy and included the following changes: hyperreflectivity of the tapetum lucidum, severe attenuation of the retinal vessels, optic disc atrophy. The physical, serological and hematological examinations were unremarkable.

Fig. 1. The 7 months old Minuet cat with marked mydriasis in ambient light. The tapetal reflection can also be noted in the left eye

The cat was premedicated using a solution of DKT (1 ml dexmedetomidine +1 ml ketamine + 1 ml butorphanol) administered at a dose of 0.04-0.06 ml/kg intramuscularly. Induction was achieved using propofol (2 mg/kg) and maintenance using Isoflurane 1.5% (Anestheran, Romania). Topical anesthesia was induced using 0.4% oxybuprocaine hydrochloride (Benoxi 0.4%, UnimedPharma, Slovakia) (2).

The cat underwent ERG examination using the Handheld Multispecies ERG system (Dog Diagnostic Protocol, HMsERG, RetVet Corp, USA) after maximal

pupillary dilation was obtained by applying 1% Tropicamide (Tropicamida 1%; Rompharm, Romania) and phenylephrine 10% (Fenefrin 10%; UnimedPharma, Slovakia) three times, with a 10 minutes interval between instillation of the drops. The ERG procedure was performed according to the current guidelines (2, 5).

Genetic testing of the Minuet cat was declined by the owner, but the patient's parents were confirmed to be carriers (N/PRA-pd) of the AIPL1 gene variant (577C>T mutation) (Fractal Bio – Veterinary Genetics Laboratory, Russia).

Results and discussions

Four different forms of PRA have been reported in the domestic cat: an autosomal dominant early-onset cone-rod dystrophy (rdy, a mutation in CRX gene manifested in Abyssinians), an autosomal recessive late-onset rod-cone degeneration (rdAc, a mutation in CEP290), an autosomal recessive early-onset rod-cone dystrophy (a causal variant in AIPL1 detected in Persians) and an autosomal recessive PRA in Bengal cats (8, 9, 10, 11, 12, 13). In Persian cats the disease occurs with complete retinal degeneration at 16 weeks (7, 15).

Since Persian cats were used in the foundation of this recently developed breed, the Minuet cat has an obvious risk for progressive retinal atrophy.

Based on the owner's observations, the patient seemed to have had vision abnormalities at 4 months of age, when it was bumping into objects.

Menace response, cotton ball, visual placing and maze test suggested complete blindness, both in scotopic and photopic conditions. Fundus examination revealed signs of retinal degeneration: tapetal hyperreflectivity consistent with retinal thinning, retinal vascular attenuation, small and dark optic nerve head (Fig. 2).

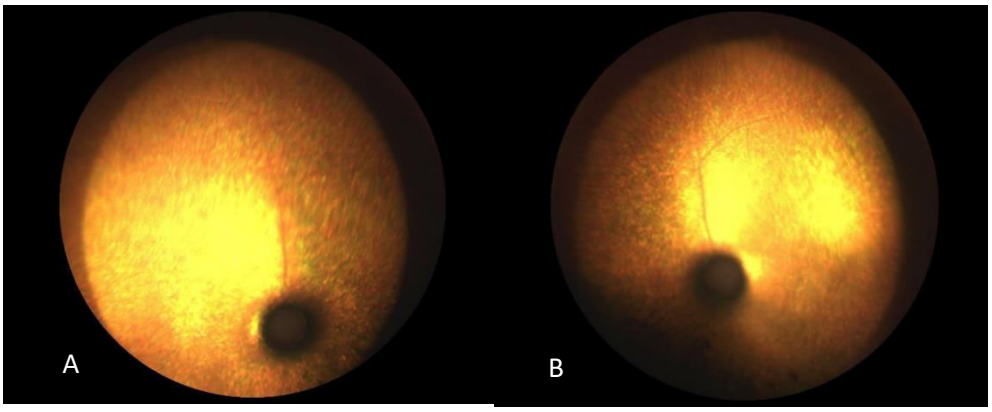

Fig. 2. Color fundus photograph of the right eye (A) and of the left eye (B): diffuse vascular attenuation, small, dark optic nerve head and tapetal hyperreflectivity consistent with progressive retinal atrophy

To confirm the fundoscopic findings consistent with PRA in this Minuet patient, an ERG was performed (Fig. 3). We used Dog Diagnostic Protocol, which is considered to be a complete protocol. ERG scotopic and photopic responses were non-detectable, which confirms the severe retinal disease, affecting the function of the photoreceptor layer (Fig. 4). The ERG also confirmed the findings of the chromatic pupillary light reflex, which indicated a presence of intrinsic melanopsin-mediated pupillary light reflex activity induced by high light-intensity and an absence of rod-cone-mediated PLR elicited by low light intensities in animals with retinal disease (6, 7).

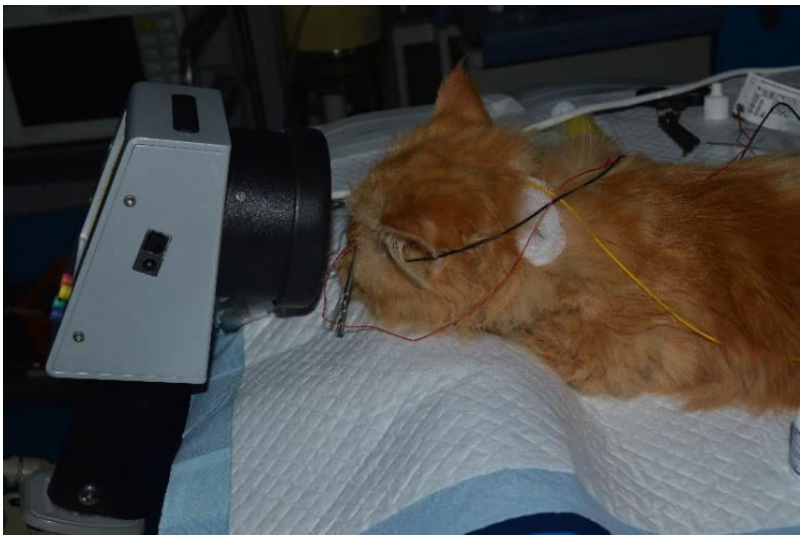

Fig. 3. ERG procedure using the HMsERG device

Genetic analysis of the patient was not possible. Both patient's parents had normal vision, but were carriers (N/PRA-pd) of the AIPL1 gene variant (577C>T mutation), which suggests that this could be the gene involved in this early retinal degeneration of the Minuet cat. This is consistent with the previous studies that describes an autosomal recessive, early-onset PRA in Persian cats (1, 15).

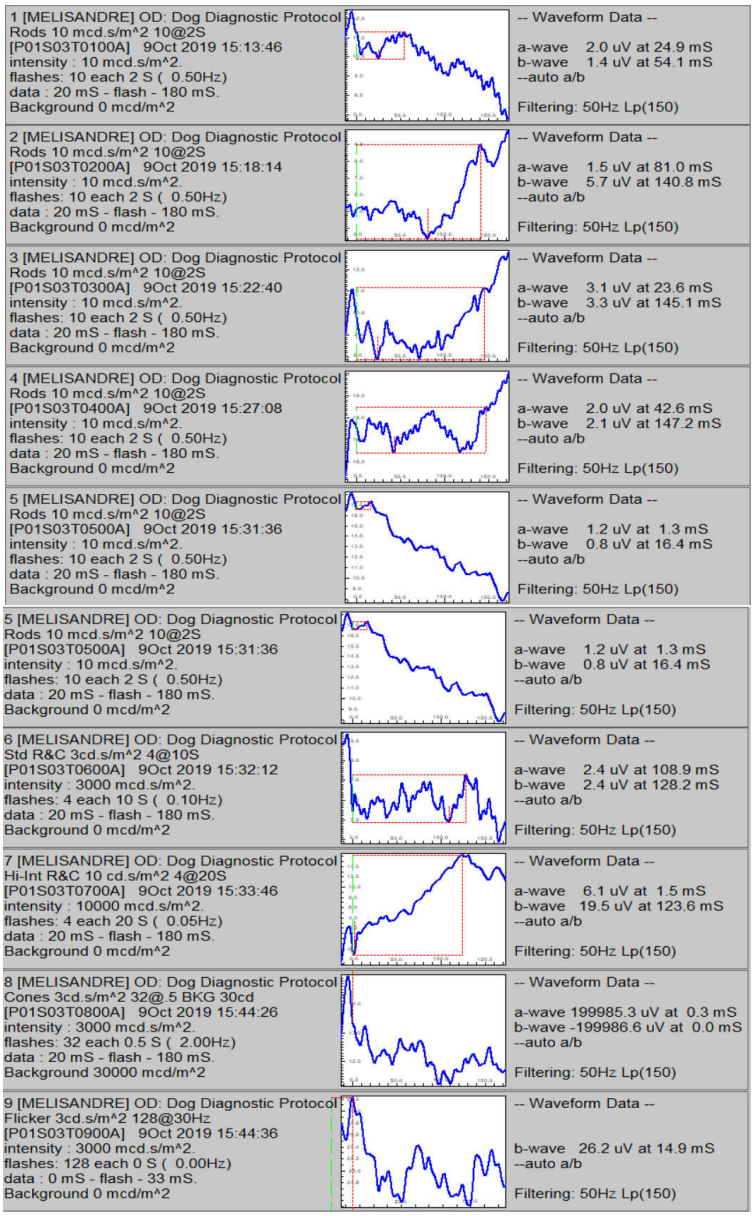

Fig. 4. ERG waveforms using the Dog Diagnostic Protocol reveals non recordable scotopic and photopic a and b waves

Conclusions

We described the clinical and electroretinographic findings of an early onset retinal disease leading to blindness before 1 year of age in a Minuet cat.

The gene causing this disease is likely to be AIPL1, which is responsible for the early onset recessively inherited rod-cone dysplasia in Persian cats.

ERG is an important tool that gives an objective evaluation of retinal function.

To our knowledge, there is the first report of early retinal degeneration in a Minuet cat in Romania.

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ENVIRONMENTAL INFLUENCE OF CHEMICAL CONTAMINANTS ON FARM ANIMALS AND RODENTS (REVIEW RESEARCH)

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Summary

The presence of chemical environmental pollutants (heavy metals) and their influence on health status of farm animals has been study in long period. We monitored the influence of chemical pollutants on rodents leaving on farms. Heavy metals has special danger for leaving systems, which react with organic molecules to change their structures and function. Heavy metals enter the body through respiratory system, digestive system and skin. The results of our many years of research indicate that there is danger of contamination of animal feed with heavy metals and their position in their body of animals, as well as a negative effect on the reproductive capacity of domestic animals. Heavy metal toxicity general leads to the formation of free radicals, inhibiting the activity of antioxidant defense enzymes as well as glutathione oxidation and the formation malonyl dialdehyde (MDA) as a marker of oxidative stress. Their toxicity stems from the tendency to form covalent bonds with sulfhydryl groups of biomacromolecules or displace certain cofactors, there by inhibiting the activity of certain enzymes. Our recommendation for industrial type farms is to reduce the risk of heavy metals. To introduce multilevel monitoring of the quality of raw materials and final products, as well as to apply adequate protectors against the toxic effect of these agents.

Keywords: farm animals, rodents, chemical contamination

This review article is aimed at presenting the results of our studies carried out in the period (2002-2020). Heavy metals pose a special danger to living systems, which react with organic molecules to change their structure and function. Heavy metals enter the body through the respiratory system, digestive organs and skin. The results of our research indicate that there is danger of contamination of animal feed with heavy metals and their deposition in the body of animals, as well as a negative effect on the reproductive abilities of animals. Heavy metal toxicity generally leads to the formation of free radicals by inhibiting the activity of antioxidant defense enzymes as well as the oxidation of glutathione and formation of maslondialdehyde (MDA) as a marker of oxidative stress from the tendency to form covalent bonds with

sulfhydrylin groups of biomacromolecules or displace certain cofactors thereby inhibiting the activity certain enzymes (3, 4, 7, 46).

Possible monitoring of hepatocytes impairments caused by ROS effects

Heavy metals are highly toxic to all living beings on Earth. They enter organisms of humans and animals via the contaminated food, water, soil and/or air. Toxicity of cadmium (Cd) and lead (Pb) is mostly due to their ability to increase reactive oxygen species (ROS) production. Redox active or redox inactive metals may cause an increase of ROS such as hydroxyl radical (HO), superoxide radical ($O_2^{\cdot -}$) or hydrogen peroxide (H_2O_2). Enhanced generation of ROS can overwhelm cells intrinsic antioxidant defenses then the consequence is “oxidative stress” (14, 35, 47). These heavy metals (Cd and Pb) are not able to induce oxidative stress by means of Haber-Weis and Fenton reactions, but they can generate ROS by indirectly mechanisms which are not completely cleared. From literature data that Cd stimulates creation ROS-a, including O_2, H_2O_2, HO (40, 43, 52).

Increased production of ROS provokes lipid peroxidation (29, 36, 38) of cell membranes, oxidation-related changes in protein molecules (23), as well as changes in DNA molecules (20). Such an action of ROS is responsible for serum albumin (SA) oxidation and fragmentation (22). The consequence of SA oxidation (18, 42) is polymerization of SA molecules and can form dimers reacting with other molecules (28). Lipid peroxidation induced by ROS action cause damages on hepatocyte membranes and destroy receptors for polySA (32, 33, 41, 42, 48, 49). Thus, the concentration of polySA increase in circulation, preventing the transfer of polySA into the hepatocytes for its degradation (53). The occurrence of polySA of increasing concentration cause formation of anti-polySA antibodies, which can be proved by immunodiffusion test against polySA prepared from sheep SA by glutaraldehyde treatment (31). The receptor for polySA on liver cell membrane functions as a binding site for in vivo polymerized albumin conferring the ability of these cells to remove polymerized molecules from the circulation as in normal organism. In this way the hepatocytes would be able to select for catabolism the polymerized albumin from the native one (33). Damage of hepatocytes membranes by lipid peroxidation is also associated by changing protein conformation and packing of phosphatidylcholine (37). Consequence of these effects are enhancement of membrane permeability which is accompanied with lactate dehydrogenase (LDH) leakage from cells increasing LDH activities in surrounding medium (26).

Heavy metals metabolism occurs mostly in the liver where Cd binds to metallothionein (MT), protein rich in cysteine (cys), with high affinity for metals. Cd is in Cd-MT complex which is transferred from the liver to the kidneys over time, then is filtered, and reabsorbed by the renal proximal tubules. Complex Cd-MT is metabolized in lysosomes, where liberate Cd ions which again binds to preexisting or newly made MT. If syntheses of MT cannot keep up with the demand of the content, unbound Cd overwhelm another defense system and Cd toxicity ensues (23, 24, 25).

In liver during heme syntheses Pb increase ROS through inhibition dehydratase of 5-aminolevulinic acid (ALA), causing accumulation of ALA which is potential endogenous free radicals sours (39). ROS is generated and by interaction of ALA and oxyHb increasing the lipid peroxidation. Lipid peroxidation altered composition of cellular membranes, change membrane permeability and enhanced its susceptibility to lipid peroxidation.

At the sheep farm Ile de France, it was noticed that the sheep lost their appetite, weight, and showed no interest in the environment. The sheep were kept free and fell from the farm pasture. During the clinical examination of the animals, the visible clinical symptoms of infectious disease were not diagnosed. The results of the testing of nutrients, grasses and soils from the same site indicated the content of heavy metals, lead, cadmium, and traces of living and arsenic.

The aim of our study was to establish the presence of polySA and SA fragments, as well as the activities of total LDH and LDH5 isoenzyme, as a consequence of ROS action, all indications of damage to the liver evident in the serum of animals previously exposed to Pb and Cd through feed. At the same time, MDA was identified as marker lipid peroxidation.

Materials and methods

Sheep Albumin and Cibacron Blue 3GA-agarose were purchased from Sigma, USA. The molecular weight standard kit was from Pharmacia Biotech, Upsala, Sweden, and other used chemicals were from Merck, Germany.

The experiments were performed on Ile de France sheep (n=20). The control group (n=10) was unexposed to heavy metals from the other ariea. The experimental group (n=10) was exposed to heavy metals (lead and cadmium) through soil and feed (soil Pb = 16.7 ± 0.02 mg/kg DS, Cd = 20 ± 1.0 μg/kg DS and feed Pb = 2.5 ± 0.02 mg/kg DS, Cd = 20 ± 1.0 μg/kg) in the course of three months. After that period, blood and blood serum of these animals was analyzed. The analysis of heavy metals in serum, soil, grass and feed samples was performed by means of atomic absorption spectrometry (Perkin-Elmer Corp., USA) after mineralization in 100A TEKATOR DIGESTOR with nitric acid, and with hydrogen peroxide added. The level of lipid peroxidation (LP) was assayed as thiobarbituric acid reactive substances (TBARS) in the red blood cells (51). The Hb concentration was determined by the cyanmethemoglobin method (13).

Blood was taken from the *jugular vein* with and without anticoagulans. After coagulation the serum was centrifuged at 3000 rpm and kept at -20°C until testing. Before freezing amount of serum was separated, and immediately used to assay the activity total LDH (EC 1.1.1.27) and activity LDH_5. Total LDH activity was carried out the by direction reduction of pyruvate to lactate was measured in Tris pH 7.4, 60.00 mM, containing 1.0 mM pyruvate and 0.18 mM NADH, by UV kinetic method. LDH_5 activity was determined in same buffer mixture with 2.0 M urea as an inhibitor (2, 27).

The PAGE electrophoresis was performed in gel concentration of 8 g/dL in

non-dissociated discontinuous buffer system (19). SDS-PAGE was carried out in gel containing 10 g/dL acrylamide (30). The electrophoretic analyses were performed on a vertical device MINI VE HEFFER.

The presence of polySA in the serum was determined by double immunodiffusion in 1.5 g/dL agarose gel in 16 mmol/L barbital buffer, pH 8.4 against prepared polySA. The preparation of sheep polySA was performed by glutaraldehyde treatment, of sheep albumin (31).

SA fragments were isolated by affinitive chromatography on Cibacron Blue 3GA-agarose mini column (0.9 x 4 cm), using 20 mmol/L sodium phosphate buffer pH 7.2 for equilibration. Elution was performed using three solutions: I 20 mmol/L sodium phosphate buffer pH 7.2; II 1. 5 mol/L NaCl in 20 mmol/L sodium phosphate buffer pH 7.2; III 5 mol/L urea. The absorbance was measured at 280. The results are expressed as arithmetical mean values ($\overline{X}$) ± standard deviation (SD). The data were analyzed by Student's t-test.

Results and discussions

The heavy metals content in the blood serum of the experimental group was analyzed, showing an increased in Pb and Cd concentrations (Pb = 3.61 μmol/L, Cd = 53.40 nmol/L), compared to the control group.

Total enzyme activity LDH in blood serum of the exposed sheep (1176 ± 63 U/L) were significantly higher ($p< 0.001$), than those in the control group (352 ± 59 U/L). LDH_5 isoenzymes from the sheep blood serum, are presented in Fig. 1.

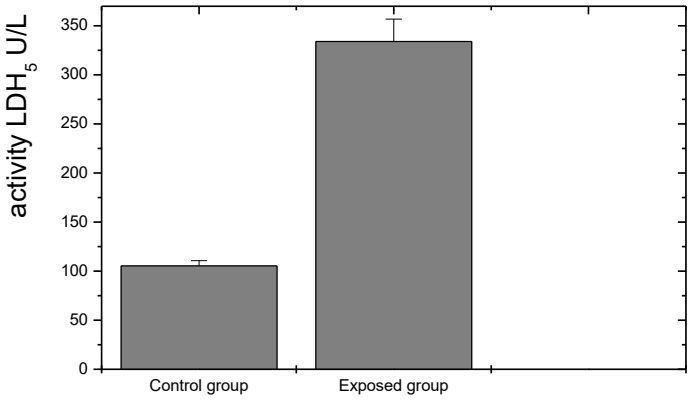

Fig. 1. LDH_5 isoenzymes activities in sheep blood serum

The activities of LDH_5 isoenzymes in serum of the exposed sheep (334 ± 23 U/L), were significantly higher ($p< 0.001$), than those in the control group (115 ± 15 U/L). MDA concentration was significantly increased in the erythrocytes of sheep

(16.95 ± 6.14 nM MDA/g Hb) after three months of taking food from a contaminated site heavy metals ($p<0.0001$) in relation on control group (0.56 ± 0.21 nM MDA/g Hb), (Fig. 2).

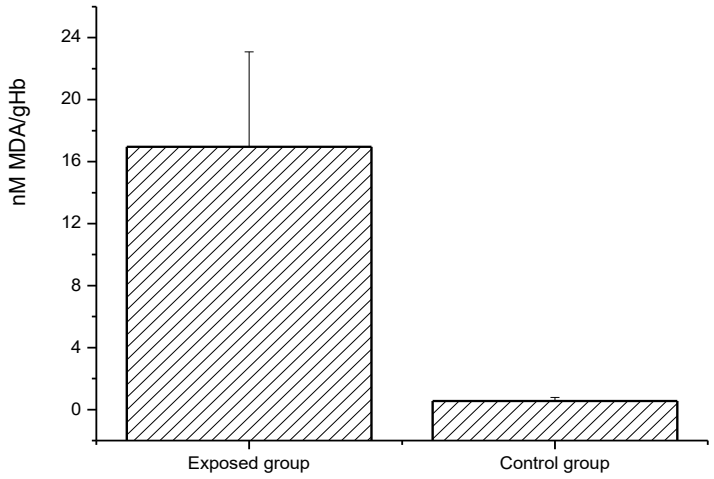

Fig. 2. The content of MDA in erythrocytes in exposed and control group of sheep

All blood samples were examined for polySA presence (Fig. 3).

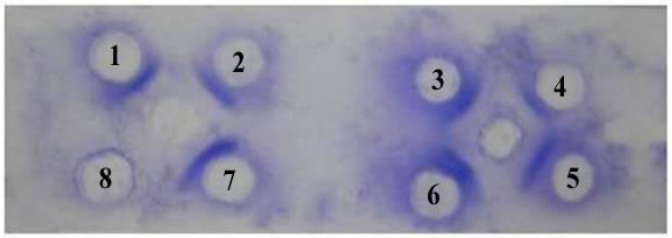

Fig. 3. Immunodiffusion test of polySA

Representative immunodiffusion reaction patterns of serum samples of exposed sheep (wells 1-7) in the control group (well 8) and central wells containing prepared sheep polySA.

The appearance of precipitin lines shown in Fig. 3 indicate the presence of polySA in serum of animals exposed to heavy metals.

Electrophoretic patterns of serum proteins of the exposed and control sheep group, and standard proteins investigated by PAGE are presented in Fig. 4.

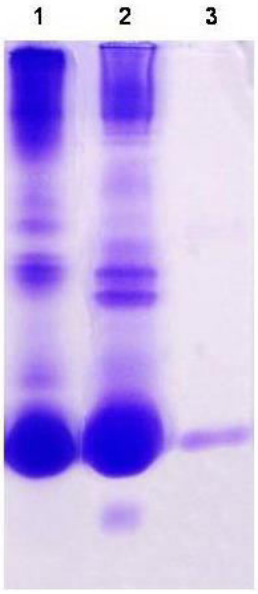

Fig. 4. Representative electrophoretic patterns of serum proteins of sheep

Representative PAGE patterns of the serum proteins in exposed sheep (lane 2), in control (lane 1) and standard proteins (lane 3).

In the PAGE protein patterns of the exposed sheep a protein fraction migrated faster than did the albumin fraction (Fig. 4 lane 2), but in sheep serum in the control group such protein fraction was not revealed (Fig. 4 line 1). The occurrence of this protein fraction was probably due to ROS effects on SA, initiating fragmentation. For confirmation, SA fragment formation in exposed sheep serum affinitive chromatography on Cibacron Blue was performed. Elution profile of exposed sheep serum is presented in Fig. 5.

Elution was carried out with buffers I, II and III and three peaks were eluted. All the obtained peaks were analyzed by SDS-PAGE. SA and SA fragments were eluted in peak 2 which was proved by molecular weight determination, using the molecular weight standard protein kit. These results are presented in Fig. 6.

SDS-PAGE protein patterns on Fig. 6 of peak 2 exhibited the presence of SA and several protein bands with molecular weight from 28 kD to 48.5 kD, indicating that SA was decomposed in fragments.

Heavy metals manifest their toxic effects by provoking oxidative stress, being their oxidation processes consequence. The ROS effect on proteins is mainly the action of hydroxyl radical (23, 24) on aminoacids in polypeptide chain. It has been proven that the hydroxyl radical mostly affects tryptophan, tyrosine and cysteine by

oxidizing them, which cause modification in the polypeptide chain with subsequent formation of fragments (22), which may behave like native molecules (Fig. 4), due to co-precipitation with the entire SA molecule. Therefore, sheep blood serum was analyzed by affinitive chromatography for separation SA with coprecipitated SA fragments from SA Elution profile obtained by affinitive chromatography (Fig. 5) of serum of the animal exposed to Cd and Pb indicate that peak 2 contains SA fragments, as well as molecules SA. This we proved by the SDS-PAGE analysis of the peak 2 (Fig. 6). Namely, results of the SDS-PAGE electrophoretic analysis of the peak 2 shows the presence of SA and SA fragments which are higher mobility proteins than SA with molecular weight 28-48.5 kD (Fig. 6 lane 1). The blood serum in the sheep control group was simultaneously analyzed, and after the affinitive chromatography of serum of these animals in the peak 2 by the SDS-PAGE electrophoretic analyses, only the presence of SA, and not the SA fragments, was proved (Fig. 6 lane 2). Protein modification by ROS-induced fragmentation in vitro, as well as the use of protectors against ROS effects, were examined by Mayo et al. (54) detecting specific cleavage of protein molecules. Oxidatively modified proteins further may spontaneous fragmented and cross linking (34, 45).

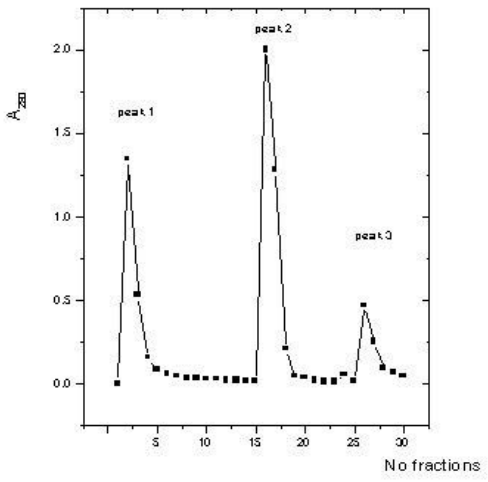

Fig. 5. Elution profile of exposed sheep serum

Representative SDS-PAGE protein patterns isolated from sheep serum by affinitive chromatography peak 2, are presented in Fig. 6 lanes from left to right. Lane 1 peak 2 from sheep serum of exposed animals. Lane 2 peak 2 from sheep serum of control animals. Lane 3 standard molecular weight markers.

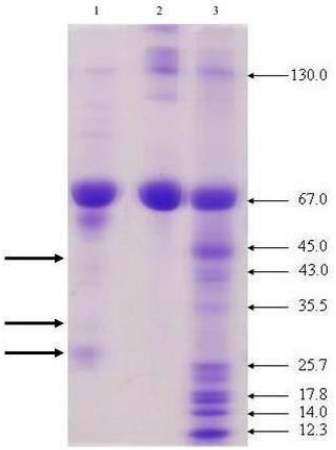

Fig. 6. Representative SDS-PAGE protein patterns of peak 2

Heavy metals thus Cd and Pb metabolism mostly occurs in the liver. The polySA receptors may damage (31, 42, 49), by various agents also by the ROS actions through lipid peroxidation of hepatocyte membranes (44), intravascularly formed polySA enhance in circulation. SA contains a free –SH (thiol group), and is a ROS action target, forming intermediary SA radical can riect with hemoglobin (Hb) forming dimmers Hb-SA and SA-SA types (42). We have tested blood serum for the presence of SA-dimmers and polySA in animals exposed to heavy metals, as well as that of the control group animals (Fig. 3). PolySA was detected by double immunodiffusion (31) in the blood serum as a result of the ROS effect on SA and hepatocyte membranes. The liver damage affects the ability of hepatocytes to remove polySa from circulation and rising its level in serum. It is proved that on liver cell membrane existed specific receptors for polySA (31, 50, 41), which involving transferring polymerized SA in to the cells to metabolism. Consequence of the liver damage breakage the specific receptors conferring polySA to liver cells, affecting the ability of hepatocytes to remove the polySA from the circulation, rising its level. Having new antigenic site induce formation specific anti-polySA antibodies. Using immunodiffusion test antibodies can precipitated by polySA formed by glutaraldehyde treatment standard SA or isolated SA from sheep serum (31). There evidence about ROS effects on hepatocytes membrane, (26)**,** study mechanism of Cd induced cytotoxicity on isolated liver rats' cells. Mechanism of this effects are associated with cellular acidification which stimulate enhancement production of H_2O_2 causing permeabilities changes of plasma membranes link to subsequent extensive breakage (26) found that Cd immediately cause acidification and later alkalinization accompanied with loss of LDH activities in the cell. Our findings of LDH activities in blood serum of exposed animals to Cd and Pb indicate significantly

increasing activities of total LDH as well as LDH_5. In human polymerized SA is detected in sera of patients with liver disease is specially hepatitis cause by HBV (hepatitis B virus). Numerous investigators have reported that polymerized human albumin and HBV have receptors on hepatocytes surface. These receptors can bind HBV and polymerized albumin and are important for the attachment of HBV to hepatocytes and entrance of virus into the cell (1, 2, 21, 50). MDA level may be use as marker of lipid peroxidation and as well as oxidative stress in regard to ROS (11, 17). Our findings indicate significantly increased the level of the MDA in erythrocytes of exposed group of sheep comparison with control one.

Results of our experiments have confirmed the presence of polySA, SA fragments as well as an increased total LDH and LDH_5 activities in serum of those animals exposed to heavy metals. We are of the opinion that these findings may serve as parameters of ROS in hepatocytes impairment monitoring.

Environmental influence on swine

Sound health of pigs is a prerequisite for good reproduction, that is, profitable production. Pig health may be improved in order to achieve the highest possible production and it depends on conditions of their keeping, care, feeding, health control and health care (8, 9, 10).

Lead content in the studied feed samples was 2.0 mg/kg dry substance; cadmium content was 0.22 mg/kg dry substance, while mercury content was 0.0035 mg/kg dry substance. Five boars used for reproduction that consumed the analyzed feed underwent semen analysis in order to determine presence of heavy metals using the method of absorption spectrometry. The obtained results indicated that the metals were transported into the reproductive organs, with the highest level of lead 0.36 mg/kg, cadmium 0.0013 mg/kg and mercury 0.0021 mg/kg. None of the above-mentioned boars underwent cytogenetic analysis. Based on the obtained results it may be concluded that detection of carriers of the chromosomal aberrations is of the great importance in artificial insemination programs. Identification of the carriers of the obscured anomalies is an important task in selection of the breeding animals. The results of the study indicate the risk of feed contamination by heavy metals and their depositing in the boar organisms. We may recommend to the industrial type farms to reduce the risk of heavy metals by introduction of multi-level monitoring of raw material and finished product quality and application of the appropriate protectors against toxic effects of the agents (5, 6).

The results indicate that is danger to contamination of animal feed with heavy metals and their deposition in the body of animals as well as reproductive capacity of boars. In order to reduce the risk of using seeds contaminated with heavy metals, it is necessary to perform an analyses of their presence. Heavy metals cause significant metabolic changes, disrupt biological systems, reduce body weight gain and the mass of individual organs. There are also differences in the accumulation of heavy metals and increased mortality depending on the age of the individuals. Heavy metal toxicity generally leads to formation of free radicals by inhibiting the activity of antioxidant defense enzymes as well as the oxidation of glutation and the formation

of MDA as a marker of oxidative stress. The toxicity is also due to the tendency to form covalent bonds with sulfhidryl groups of biomacromolecules or displace certain cofactors which inhibit the activity of certain enzymes (7, 8, 9, 10).

Environmental influence on rodents

Different storage conditions for agricultural products and the efficacy of environmentally-safe substances were analyzed to formulate a biosecurity standard. Hygienic conditions, the most important such indicator in any facility, are crucial for controlling house mouse (*Mus musculus* L.) populations. It is often impossible to remove all food sources from a facility and its surroundings. A preventive biosecurity programe based on HACCP principles aims to prevent the infection of primary biological vectors in any agricultural storage facility. Our data show the efficacy of sodium selenite and cellulose and their applicability in biosecurity plans considering their application methods at critical control points (15, 16).

Conclusions

Veterinary profession has a very important role in livestock breeding, animal health protects people and the environment. Every day the question how to produce more animal products, but also healthy food intended for human consumption. Therefore, the health care farm animals using more preventive and therapeutic measures less able to fulfill the concept which will be represented at the same time profitable production, and degree control over of toxic agents in the responsibility veterinary professions involved.

Our recommendation for industrial-type farms that need to act to reduce the risk of side effects of heavy metals, for the introduction of multiple-quality monitoring of raw materials and finished products, and implementation of adequate protector of the toxic effects of these agents.

Acknowledgements

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USE OF DACARBAZINE AS PHOTOSENSITIZER IN THE TREATMENT OF WALKER 256 CARCINOSARCOMA

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Summary

The method of selective Photostimulated Chemotherapy (PSChT) is used to increase the concentration of a specific substance in the malignant tissue. Depending on the pH of the solution, there may be different tautomers of a drug. In this study, UV-Vis absorption spectroscopy was used to obtain new information on the influence of pH on the absorption spectra of Dacarbazine in order to use this compound as a potential agent in Photostimulated Chemotherapy procedures. The changes that occur in the absorption spectra, due to the change in pH values, have been attributed to the presence of protonated and deprotonated DTIC species present in acid and alkaline solutions, respectively. In order to confirm the positive photodynamic potential, preclinical DTIC-PSChT experiments were performed on laboratory animals (Wistar rat) inoculated with Walker 256 carcinosarcoma, and the results was in favor of this protocol. The aim of this paper is to highlight the effectiveness of Photostimulated Chemotherapy and Dacarbazine in Wistar rats inoculated with Wlaker 256 tumor.

Keywords*:* dacarbazine, photostimulated chemotherapy, carcinosarcoma, Walker 256 tumor

It is to be expected that 14 million of people develop cancer each year, and this number could increase to more than 21 million until 2030. This disease is responsible for almost one in each six deaths worldwide. Each year, 8.8 million of people died from cancer especially in low-income countries (1). Among the causes of death by cancer is cachexia, responsible by 20% of death. This complication in oncologic patients cooperate to a worse prognostic, lower survival, alterations quality of life, deterioration in functional capability, as well as significantly contribute to toxicity induced by chemotherapy (2, 3).

About 30% of human patients diagnosed with breast cancer develop metastatic brain tumors making them very difficult to investigate and treat due to the specific nature of this organ (10, 17). However the animal models are important tools to study this disease and develop drug therapies (11, 12, 18).

Walker-256 Carcinosarcoma appeared spontaneously in the mammary gland region in a pregnant female rat, discovered by Walker in 1928 (4, 5). Initially, the tumor was diagnosed as Adenocarcinoma which includes a population of

sarcomatoid cells. The carcinomatous variant selected during the passages is composed of cellular structures supported by a stromal tissue made up of collagen fibers and reticulin. The tumor is well vascularized with dilated vessels near the numerous foci of necrosis. The sarcomatoid variant has fusiform cells surrounded by reticulin and collagen fibers. The sarcomatoid variant has fusiform cells surrounded by reticulin and collagen fibers. The percentage of tumor grafts uptake is 80-90% with seasonal variations. The tumor is maintained by subcutaneous grafts in Wistar rats. It rarely metastasizes and the invasion of the loco regional nodes takes place in the terminal phase of tumor growth when the phenomena of tumor necrosis are accentuated. The ascitic variant was obtained by Waren and Gates (1936), without being maintained in this form. Agostino and Clifton (1959), Planisec (1965), Comisel (1967), manage to obtain the ascitic conversion of the solid tumour which is maintained even today as ascitic tumour through serial passages in the rat (6).

The aim of this paper is to propose an innovative approach to the classical therapeutic protocol of this tumor, using, along with classical monochemotherapy, Dacarbazine, and the method of Photostimulated Chemotherapy (DTIC-PSChT). The mode of action of DTIC is still uncertain, but most sources state that this drug works similarly to other cytotoxic drugs classified as "alkylating agents". These agents stop cancer cells from dividing by coupling with the chains of the cell's genetic material. This makes the double helix inseparable, which is necessary in DNA replication. As a result, the cells can no longer divide and they die (7, 8).

Data from the literature show that, depending on the pH value of the solution, a tautomer of this drug with different biological activities is expected to occur. Thus, it was found that the toxicity of Dacarbazine is dependent on light and is greatly affected by the pH of the environment (9).

It is known from the literature that the starting geometries used for structure optimizations were obtained from X-ray diffraction data reported by Freeman and Hutchinson. These data show the existence of two tautomers in the asymmetric unit (Figure 1). The difference between them is related to the position of the second hydrogen atom in the imidazole ring. In one molecule, the protonated nitrogen of the imidazole ring is adjacent to the triazene group, while in the other, it is adjacent to the carboxamide group (13, 14).

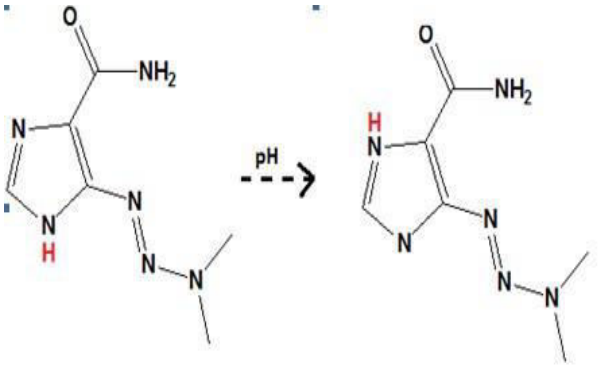

Fig. 1. Tautomerization

Materials and methods

1. Physico-chemical determinations

The absorption spectrum of Dacarbazine and, subsequently, its photodynamic potential, as a function of pH, were investigated, correlating the physical results with the preclinical ones in a Photostimulated Chemotherapy protocol that uses a mercury vapor lamp as an irradiation source (15, 16). Thus, for spectroscopic measurements, Dacarbazine Lipomed 200 mg powder and used as such. UV-VIS spectra were recorded primarily for aqueous solutions at various concentrations (10^{-4} M to 10^{-6} M) and at normal pH and, secondly, at a concentration of 10^{-5} M for different pH values of 7 and 13, respectively.

The desired concentrations for measurements were obtained from an initial solution with a concentration of 10^{-2} M. For the preparation of aqueous solutions, double distilled water was used. The samples were stored at room temperature in plastic containers wrapped in aluminum foil, the pH of the solutions was changed by adding small amounts of 1% sodium hydroxide (NaOH) solutions. UV-VIS spectra were recorded using a double-beam spectrophotometer equipped with a Perkin Elmer LAMBDA 25 silicon photodiode detector. The samples were placed in 1 cm long quartz dishes in a solution of double-distilled water. The spectra were recorded in the range 190 -1100 nm. The dose used is 4 mg/animal except the control group. For subjects that have been treated using the photostimulated chemotherapy method, the established dose followed by irradiation 5 minutes course has been applied.

2. *In vivo* experimental model

The *in vivo* experimental model consisted in the subcutaneous transplantation of a solid Walker 256 tumor on a number of 15 rats divided into 3 equal groups, respectively: group I - control (untreated), group II - experimental (treated with DTIC) and group III - experimental (treated with the DTIC-PSChT

method).

In order to determine the cytostatic effects, tumor fragments were collected from these animals from which histological slides were made. For this purpose, tissue biopsies (fragments of tumors, lungs, liver, spleen and lymph nodes) were shaped, fixed for 24 hours in 10% formalin buffered solution, embedded in paraffin and sectioned to a thickness of 2-3 μm, with a manual microtome RM 2125 RT (Leica Biosystems). The sections obtained from each sample were automatically stained with Hematoxylin-Eosin (H&E).

The histopathological examination aimed to identify tumor formations in different tissues, their morphological aspects and to identify the degree of intratumoral necrosis related to the total surface of the tumor**.**

Results and discussions

1. Spectroscopic determinations

The experimental absorption spectrum has two peaks: one at 328 nm, representing the electronic transition between the fundamental level and the first allowed excited state, and the second at 236 nm, attributed to a transition between it and the second allowed excitation state. Both absorption peaks retain their shape and position when the DTIC concentration in the solution changes.

UV-VIS spectra of DTIC water solutions at different pH values were recorded in the 2-13 pH range. As pH values increase, the spectra show considerable changes. One can easily notice a "red shift" of the bands associated with the first and second excited states. Thus, the first band shows a displacement from 222 nm at pH = 7 to 248 nm at pH = 13. The second band (the one of interest) passes from 328 to 342 nm under the same conditions (Fig. 2), thus becoming resonant with the photostimulatory radiation emitted by the equipment used (Fig. 3).

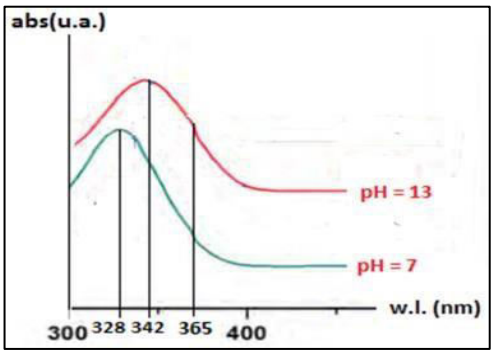

Fig. 2. DTIC absorption depending on pH

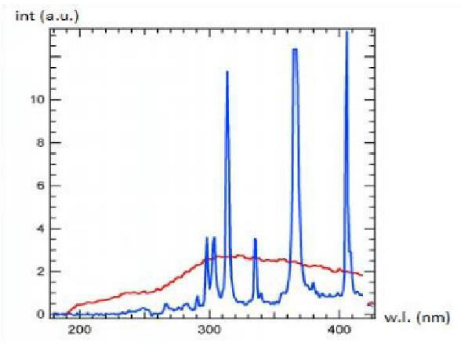

Fig. 3. Photostimulation spectrum

2. *In vivo* experimental determinations

In this graph (Fig. 4) shows considerable decrease in tumor volume following treatment with decarbazine and photostimulation. For this consignment consisting of 5 animals, the tumor volume records a steady decrease, in the first measure of 1.5 mm and on day 73 by a 0.05 mm, thus revealing the strong antitumor effect of the treatment.

The increase in the tumor volume following classic treatment with decarbazine is observed in Fig. 5. For this consignment made up of 5 animals, the tumor volume records a slight increase until day 37 and subsequently a considerable increase in day 73.

In this graph, there is a considerable increase in the average of the tumor volume of this blank lot consisting of 5 animals, observing how the tumor volume reaches 800 mm on day 73 (Fig. 6).

The effectiveness of treatment with dacarbazine and photostimulation is observed in Fig. 7, where the tumor volume recorded a decrease in the measurements compared to the classical treatment with dacarbazine to which the animals had an increase in tumor volume. Also, the impressive increase in tumor volume for the control group is observed, to which the animals have not received any treatment.

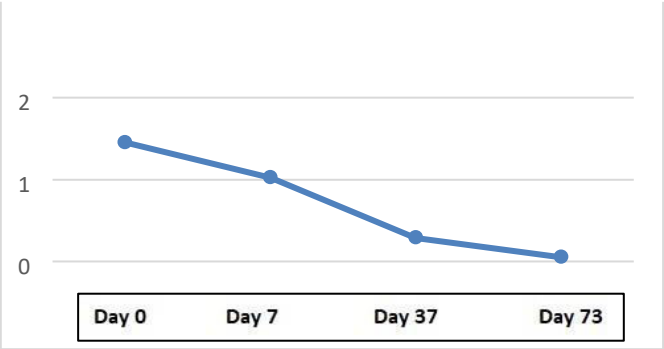

Fig. 4. Evolution of tumor volume following treatment with dacarbazine and photostimulation

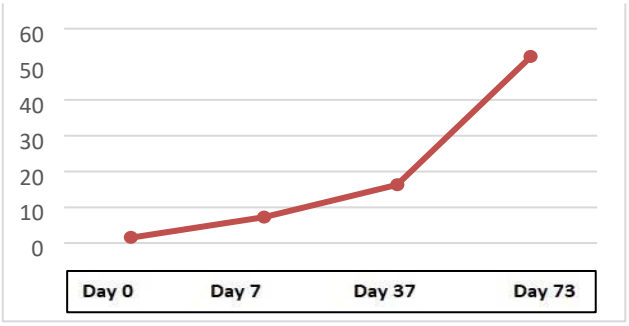

Fig. 5. Evolution of tumor volume following classic treatment with dacarbazine

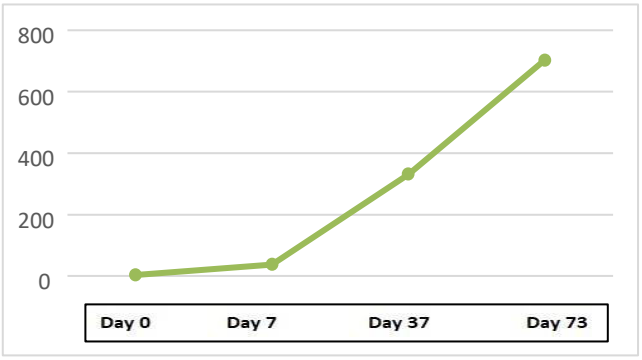

Fig. 6. Evolution of tumor volume for batch without treatment

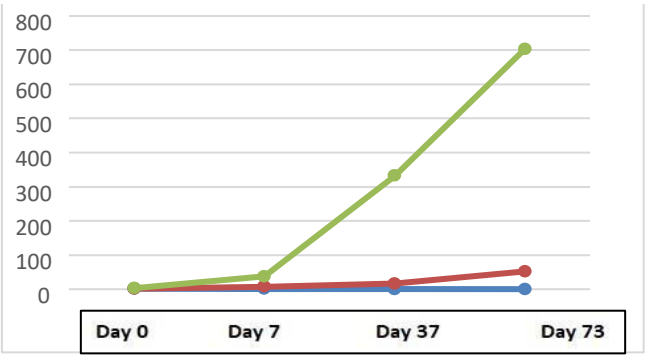

Fig. 7. Graphic representation of tumor volume evolution for the three lots

In Fig. 8 it is shown the effectiveness of treatment with dacarbazine and photostimulation, where the tumor volume recorded a decrease in the measurements compared to the classic treatment with dacarbazine to which the animals had an increase in tumor volume.

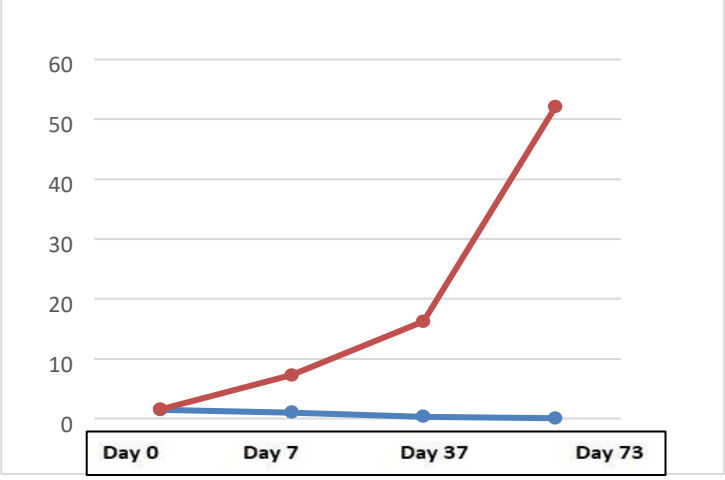

Fig. 8. Graphic representation of the evolution of the tumor volume for loads with the classic treatment with dacarbazine and treatment with dacarbazine and photostimulation

3. Macroscopic aspects of the tumor's evolution

In Fig. 9A it can be seen that the treatment with dacarbazine and photostimulation has a clear superiority over the classic method of chemotherapy but also over the control, which has a considerably increased tumor volume.

In Fig. 9B it can be observed visible traces of necrosis and an increased volume of the tumor compared to the method of chemotherapy and photostimulation.

In Fig. 9C it is shown an animal from the control group, which received no treatment. The speed of tumor mass growth is explained due to non-application of the treatment schemes used in animals from the figures 9A and 9B.

Fig. 9A. Animal treated with dacarbazine and photostimulation

Fig. 9B. Animal treated with classical chemotherapy with dacarbazine

Fig. 9C. Animal from the control group (no treatment)

4. Histopathological aspects

Section through the primary tumor where cellular details are observed with a solid appearance with marked pleomorphism, marked anisokaryosis, small cytoplasm, occasionally moderate and discreetly vacuolized, the stroma is a fine vascular conjunctiva and occasionally to the periphery of the tumor where the pseudo-capsule of collagen fibers is observed, lymphocytes and rare plasma cells are also present. Atypical mitoses are numerous, averaging 8-12 to a target of 40.

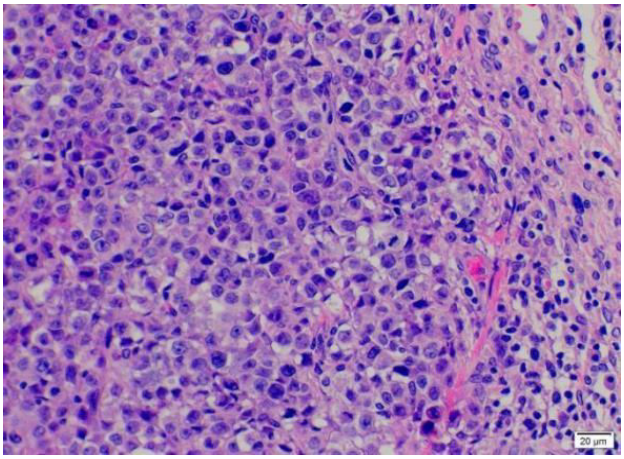

Fig. 10. Walker 256 Carcinoma, primary tumor (solid variant that was used for inoculation). Objective 20, H&E stain

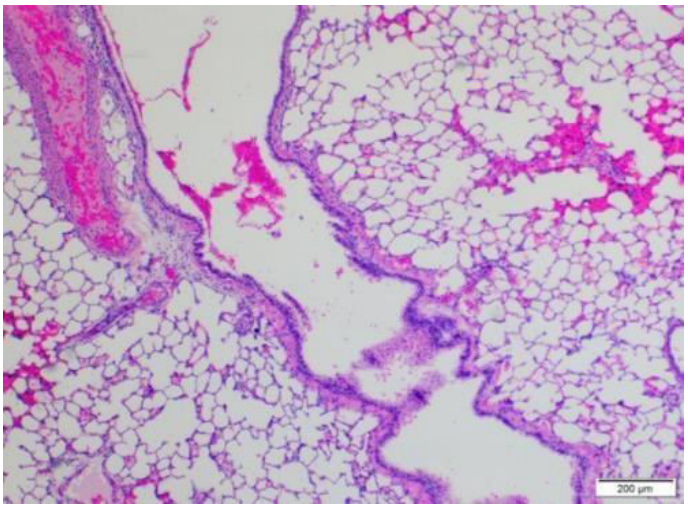

Fig. 11. Normal lung (without any presence of tumour formation) from animal treated with dacarbazine and photostimulation. Objective 5, H&E stain

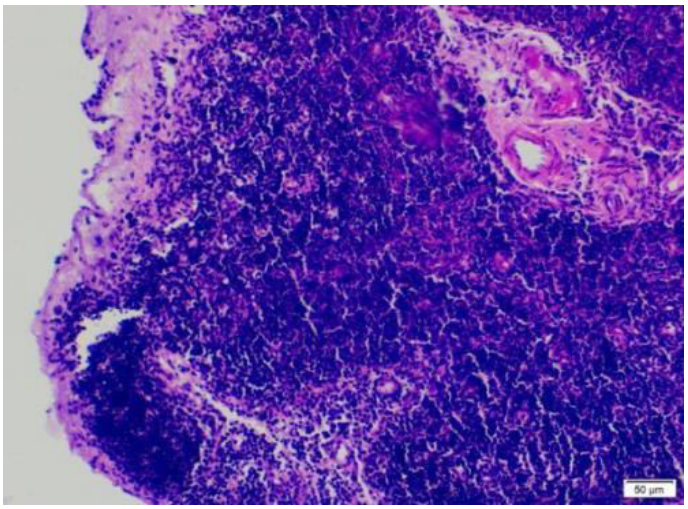

Fig. 12. Normal lymph node from animal treated with dacarbazine and photostimulation. Objective 10, H&E stain

Cell density, lymphoid population is abundant, with follicle formation and the presence of lymphoid cells in the subcapsular and medial sinuses, without

neoplastic aspects.

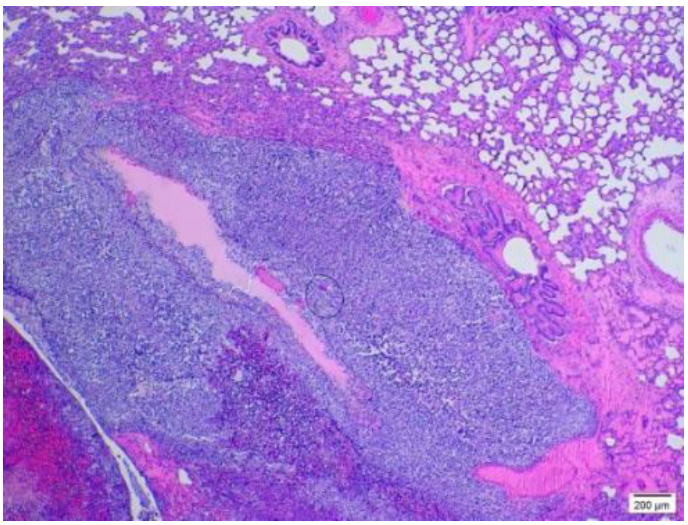

Fig. 13. Lung with metastasis from animal treated with classical chemotherapy. Objective 2, H&E stain

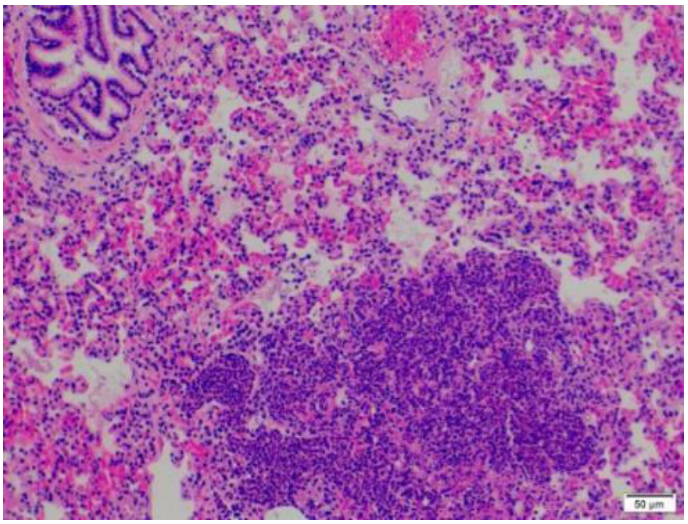

Fig. 14. Lung with metastasis from animal in the control group (no treatment received). Objective 10, H&E stain

A rich cellular neoplastic formation is observed, which occupies about 40% of the examined lung section. It is unencapsulated, with a tendency to infiltrate, compressing the adjacent lung tissue. The neoplasm is richly cellular, with a morphology similar to that described in previous sections in rats where the tumor developed.

Section through the lung with metastasis, a bronchiole is captured in the upper left corner and the lower right side with tumor population. Target is 10, hematoxylin-Eosin staining is an unencapsulated nodule, with increased cell density, spindle-shaped round cells with characteristic features for the tumor described above, lung with metastasis.

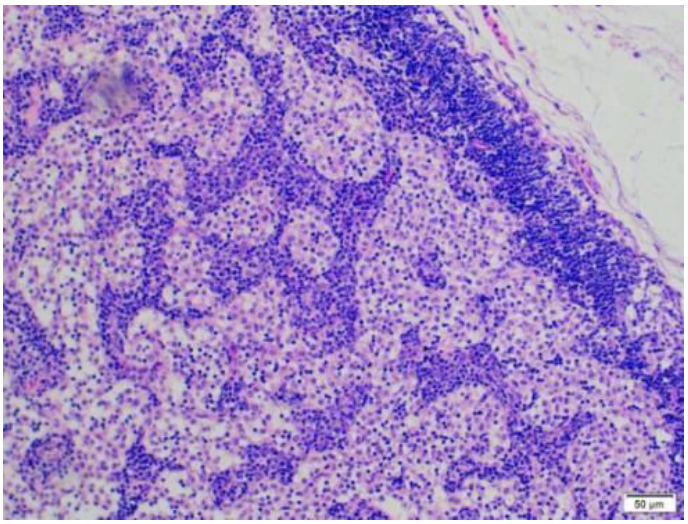

Fig. 15. Lymph node with metastasis from animal in the control group (no treatment received). Objective 10, H&E stain

Lymph node section with metastasis in the subcapsular sinuses and slightly in the medullary sinuses, are enlarged in volume, expanded by a polygonal cell population, round cells that compress the resident lymphocytes suggesting a lymph node metastasis.

Conclusions

From the analysis of the UV-VIS absorption spectrum of Dacarbazine, it is observed that it may have a positive photodynamic potential in the conditions of optical irradiation in the UVA field.

In order to optimize the absorption of the radiation emitted by the Hg vapour lamp, used in the standard PSChT procedure by this drug, it is necessary that the administered solution has a pH as alkaline as possible.

Both the graphs and the histopathological samples of Walker 256 carcinosarcoma in the three groups in the study attest to the fact that the use of the DTIC-PSChT method was significantly effective.

Following the experiment, there was an increase in the effectiveness of anticancer chemotherapy by combining photostimulation with dacarbazine, even in the advanced stages of tumors. This method could favor the reduction of cytostatic doses resulting in a reduction in side effects. The financial impact could be reduced with the combination of the photostimulation method and a low price cytostatic. The quality of life and the survival time of the subjects in the experiment were superior to the classic treatment.

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A BRIEF REVIEW OF THE DYSTHYMIC DISORDER'S MAIN ETHOLOGICAL AND PATHOPHYSIOLOGICAL COORDINATES IN DOMESTIC CARNIVORES

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Summary

The dysthymic disorder represents one of the most common behavioral pathology found in small animals, especially in companion dogs and cats, and is often clinically translated through symptoms like general unpredictable aggressive behavior, the disruption of the wake/sleep cycles and constant sound manifestations like moaning or whining. An all-encompassing definition of this behavioral disorder has not yet been established; however, it can be presented as the totality of manifestations intended to attack or produce physically injures to other individuals of the same species or different species, behavioral acts performed by threat or direct physical contact. The characteristic manifestations of the disease can be differently expressed in each patient, the common point being the superior nervous structures lesions. The early detection and correct diagnosis of dysthymia are often difficult mainly since some forms of aggression are wrongly considered to be normal in several breeds and tend to be seen as disorders only when they become directed toward the owner or are present in unusual situations. The origin of the aggressive behavior can be physiological – the innate aggressiveness of some breeds, can ethologically be induced by the owner or trainer, or it can be the result of pathological processes at the level of the central nervous system – most common being the brain lesions that are, in almost all reported cases, corroborated with a genetic predisposition or a hostile breeding environment and can lead to extremely aggressive behavioral manifestations. The aim of this review is to raise awareness on this common behavioral disturbance, by highlight the most relevant coordinates of the dysthymia in domestic carnivores in terms of pathogenicity, predisposition, ethological implications, common and uncommon clinical manifestations.

Keywords: dysthymic disorder, carnivores, dysthymia, aggressive behavior

Aggression and hyper aggression in domestic carnivores are conditions defined as any behavior that intimidates or injures a person or other animal. Roaring or biting are considered aggressive behaviors for these species. Although aggressive behavior is normal in the pack, it is generally considered unacceptable by humans. Due of the fact that humans and domestic carnivores have different values and communication systems, misunderstandings can occur due to lack of communication between the two species. A man may intend to be friendly, but the animal may perceive this behavior as a threat or intimidation.

The dominant anatomical substrate for aggressive behavior is considered to be the limbic system. The responsible components are the complex

interconnections of the nuclei in the primitive cerebral areas, with the thalamic, hypothalamic and central ones. Morphological and functional alteration of some of this structures initiates, maintains or exacerbates the aggressive behavior in domestic carnivores.

The involvement of genetic factors in the development of aggressive behavior is unanimously recognized. An essential argument in this regard is the so-called "idiopathic aggression" that is often recorded in some dog breeds like German Shepherd, Doberman or Spanish Cocker (Fig. 1). Ethological studies have shown that, in general, males are much more aggressive than females. If the aggressive behavior is innate, it is necessary to look for possible interactions or events related to situations that occurred in the environment. In these situations, it is strongly recommended to perform a neurological physical examination and also detailed laboratory investigations in order to highlight any neurological or systemic disorders (9).

Breed	Snaps, bites or attempts to bite		
	Stranger	Owner	Dog
Dachshund	20,6%	5,9%	17,6%
Chihuahua	16,1%	5,4%	17,9%
Australian Cattle Dog	9,6%	1,5%	20,6%
Border Collie	8,0%	1,8%	13,5%
Beagle	7,9%	7,9%	9,5%
Jack Russell Terrier	7,7%	3,8%	21,8%
Pit Bull	6,8%	2,3%	22,0%
Australian Shepherd	6,2%	0,6%	14,7%
Boxer	5,7%	0,0%	15,7%
Great Dane	5,7%	1,9%	9,4%
Doberman Pinscher	5,6%	1,4%	11,1%
Rottweiler	4,8%	1,0%	7,6%
Cocker Spaniel (American)	4,7%	5,6%	7,5%
Bichon Frise	4,6%	1,5%	4,6%
Airedale Terrier	4,5%	1,5%	9,1%
German Shepherd	4,5%	2,1%	16,4%
Soft Coated Wheaten Terrier	4,2%	1,9%	16,2%
English Springer Spaniel	3,5%	3,5%	17,5%
Shetland Sheepdog	3,5%	3,5%	3,5%
Akita	3,0%	1,0%	29,3%
Havanese	2,7%	0,0%	4,1%
Portuguese Water Dog	2,7%	0,0%	6,7%
Mastiff (English)	2,4%	0,8%	6,3%
Labrador Retriever	2,3%	1,7%	4,3%
Greyhound	1,6%	0,0%	1,6%
Bernese Mountain Dog	1,5%	3,0%	4,5%
Collie	1,5%	2,3%	6,8%
Rhodesian Ridgeback	1,4%	0,0%	5,8%
Poodle	1,2%	0,0%	7,7%
Golden Retriever	1,1%	0,6%	7,2%
Brittany Spaniel	0,0%	1,5%	4,5%
Siberian Husky	0,0%	1,9%	5,6%
Whippet	0,0%	1,7%	3,4%
Average	**4,7%**	**2,0%**	**10,7%**

Fig. 1. Statistics regarding dog aggressiveness in direct correlation with the breed (5)

The manifestation of the aggressive behavior can vary depending on the temperament, the environment or even the individual. Some individuals tend to respond aggressively to the slightest threat or stimulus, while others may be challenged for a long time and never try to aggressively respond. The difference lies in the individual's self-control, which can be influenced by both environmental and genetic factors. Training programs and self-control education can successfully be used in some dogs, teaching them to stop responding aggressively to various external factors. This control can be educated by using behavior modification techniques. If the chosen techniques are appropriate and correctly implemented, then how easily this control can be educated and modified depends on: the breed, the environment and the individual's temperament.

If the animal is perfectly healthy, except for behavioral disorders, then it is necessary to determine precisely the type of aggression and possibly the location

of possible lesions. Following multiple paraclinical investigations, the cause and source of a preexisting disorder can usually be located. However, the types of aggression are extremely numerous and usually associated, thus limiting the effectiveness of the curative interventions. On many occasions, especially in males, owners opt for sterilization to reduce testosterone levels and avoid aggression. However, multiple research papers regarding the behavior of castrated dogs had shown that aggression does not decrease after the procedure. The clearest behavior observed after castration in males being only the cessation of female search.

A group of scientists from the University of Arizona conducted a study with the main purpose to analyze the function and concentration of various hormones, other than testosterone, in order to find out more about this canine behavior (6). This disorder can be caused by a brain dysfunction, a chemical imbalance that causes instability, which must be addressed by the veterinarian. It is very important not to confuse canine depression with dysthymia, the two disorders having a different therapeutic approach.

Dysthymic disorder is also known as a bipolar disorder, which is characterized by drastic changes in mood, like silence before a manic or depressive state, or vice versa. Mania is characterized by a pleasant sensation, which gives affected subjects energy, associated with the obsession or possession of certain objects, people or even habits, thus preventing cohabitation between family members and generating exclusivity through aggression (Fig. 2).

It is considered to be a neurological condition, some of the theories regarding the origin of the disease including: a particular form of epileptiform manifestation, a form of "canine schizophrenia", the involvement of low levels of serotonin in the brain, or even thyroid imbalances. The dysthymic disorder can be monopolar when the behavioral changes are directed in one direction or bipolar when the mood can vary between uncontrollable joy and depression for no apparent reason (4). The clinical pathological coordinates of dysthymia in dogs are: permanently dominant attitude, for no apparent reason, "idiopathic" aggression with sudden onset, depressive manifestations, loss of appetite, lack of cooperation and loss of “playfulness”, drowsiness, apathy and lethargy, manifestations of unjustified aggression on owners or other animals, a state of fatigue and physical exhaustion.

In the experiment mentioned above, one of the hormone levels that were measured was that of oxytocin, this hormone being responsible for the emotional behavior. During the study, dogs with a previous history of aggression directed towards humans and non-aggressive dogs were selected. Blood samples were collected before and after the dogs were subjected to various tests, such as observing another dog or an unknown person. The results of oxytocin levels did not show significant differences between some dogs before and after the various tests, indicating the fact that the theories regarding the involvement of this hormone in the onset of dysthymia are, most likely, not valid (6).

Another hormone that was taken into consideration in this study was the vasopressin. Until the time of the experiment, the role of vasopressin in dogs has

never been studied, however, studies have been performed with other mammalian species. They showed that vasopressin plays an important role in aggression towards strangers. For example, a human study showed that males who received a dose of vasopressin had difficulty appreciating the friendly gestures of strangers. The data showed that there appears to be a clear relationship between vasopressin and aggression. When the levels of vasopressin in dogs with an aggressive history and non-aggressive dogs were determined, the results were different from those obtained by measuring oxytocin. Aggressive dogs had higher levels of vasopressin than non-aggressive dogs (6). These results show that there is a clear relationship between dog aggressiveness and their vasopressin levels.

In this research, to reinforce the findings, were also measured the levels of oxytocin and vasopressin in assistance or therapy dogs. In their case, a large increase of the oxytocin level was detected. Dogs that have been bred and trained to support certain people are infinitely more docile and less aggressive than dogs kept as pets (6). They also did not show a high blood concentration of vasopressin. In conclusion, the study shows how other hormones, other than androgens, have a fundamental role in canine docility or aggression.

There are many types of aggression in dogs, like: protective aggression – in this case the dog can exhibit aggressive behavior when it believes that one of their family members is in danger; possessive aggression – regarding their belongings; fear aggression - a frightened dog can become aggressive if cornered; defensive or pain aggression - motivated by fear or pain; and others. This neurological condition is quite common in some breeds like Spanish Cocker, not being characteristic or associated with a particular breed. It is also described in the American Cocker, Siberian Husky, Dobermann, Bull Terrier, German Shepherd, Golden Retriever, Saint Bernard (7).

The treatment of aggression is extremely complex, being recommended only after establishing a definite diagnosis. It is also strongly recommended to identify and exclude the circumstances that induce these behavioral disorders, in order to limit or eliminate the repetition and permanence of the aggressive. Therapeutically, it is recommended to limit the use of psychotropic agents as much as possible, this type of drugs representing the extreme alternative, used with caution only in limited cases and for a short period of time, after performing thorough clinical and paraclinical examinations (3).

In dogs, in addressing these behavioral disorders it is necessary to pay special attention to the progressive installation and the contribution of degenerative changes - attributable to the geriatric age segment. In this category, a distinct place is occupied by the hyper aggressiveness of the old dog. Dysthymia of the old dog is a disorder that evolves gradually, in two stages, with a first period corresponding to unipolar dysthymia and the second period characterized by bipolar dysthymia. The typical element of this condition is the loss of the ability to evaluate the relationship between the width of a space and the size of one's body. The old dog

with dysthymia tends to force its way through a narrow space and may become trapped, any attempt of outside help can provoke an aggressive response.

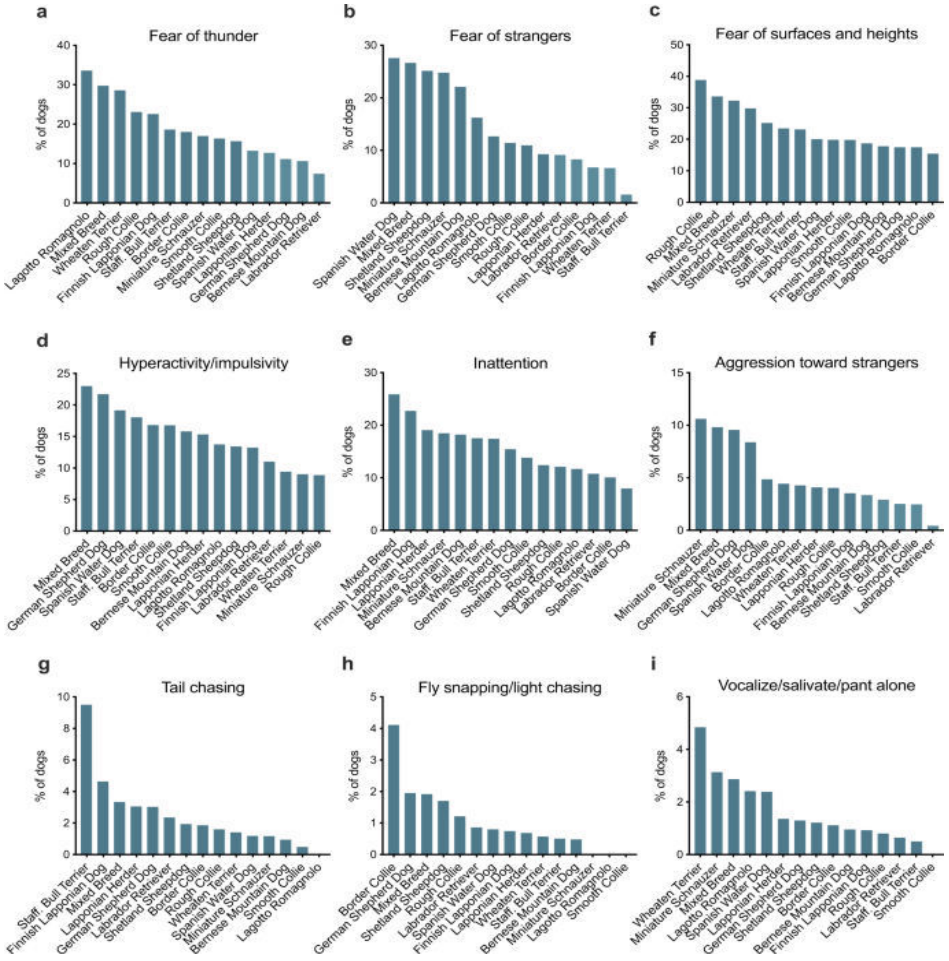

Fig. 2. The prevalence of fear of thunder (a), aggression toward family members (b), hyperactivity/impulsivity (c) and fear of strangers (d) for both sexes and six age groups of dogs (11)

The bibliographic data regarding the etiopathogenesis of the old dog dysthymia are not edifying, there are etiological hypotheses related to hypercorticism, dysthymia being found both in animals with Cushing's syndrome and in those treated for a long time with retarded corticosteroids (5). The diagnosis takes into account a number of characteristic elements, the most important being

the occurrence of the disorder in dogs over 7 years of age, clinically correlated with the inability to assess a passageway and forcing passage through the narrow space. The treatment of this syndrome is exclusively biological, the only drug that gives constant results is selegiline (Eldepryl).

The dysthymic disorder can be often seen in cats also, the most obvious and easiest to understand aggression behavior between cats occurring in unsterilized cats. As the cats mature, they begin to challenge each other for the defense of the territory. Cats that wander in the territories of other cats can end up taking part in real fights. Before the fight, the cats will have a rigid upright position, with their backs slightly raised, staring at each other. The ears will be oriented towards the back and the cat will start meowing or whistling loudly. Eventually, a cat will leave, or the fight will begin.

Aggression due to fear can occur if a cat perceives a threat and attacks if it cannot withdraw. The greater the threat, the more intense the fear and therefore the aggression with which it will react. Aggressive events appear especially if the cat cannot escape from the obstacle that causes fear.

Redirected aggression is the most dangerous aggressive behavior encountered in cats due to constant biting and extremely aggressive attacks, the consequences of which are extremely serious (2). This type of aggression occurs when a cat is aggressively agitated by another animal or a person it cannot reach - when it is a window or door between them. Unable to reach the source that causes the agitation, the cat usually takes revenge on someone else who is nearby.

Some studies examined cat breeds' differences in behavior using logistic regression and took into account environmental factors (weaning age, access to outdoors, and presence of other cats) as well as general factors (sex and age) by including combinations of these variables in the analyses (Fig. 3).

There may be a long period of time between the initial irritation and the onset of the attack, that is the reason why most owners consider this type of aggression as unpredictable or sudden. The cat will not look for someone to attack but will redirect the aggressive behavior on any person or animal that is nearby or trying to approach. This type of aggressive behavior is not malicious but is more of a reflex. Animals of many species struggle to expand or preserve their territory, and cats are no exception to this rule. Territorial aggression of cats is usually directed at other cats, but can also be directed at dogs or humans. Cats mark their territory by patrolling, rubbing their chin or spraying urine. Cats can stalk, chase and hunt the target intruder while having an offensive body position along with meowing, hissing and spitting. Some cats slowly attack their intruder, while others become very aggressive immediately after being detected.

The aggression between cats in the house is much more subtle and complex than that between cats outside. The aggressiveness of domestic cats can be so subtle that family members do not even notice it most of the time (1). The offensive aggressor will advance towards his opponent, and the defensive cat will become smaller and smaller or will decide to give up the fight.

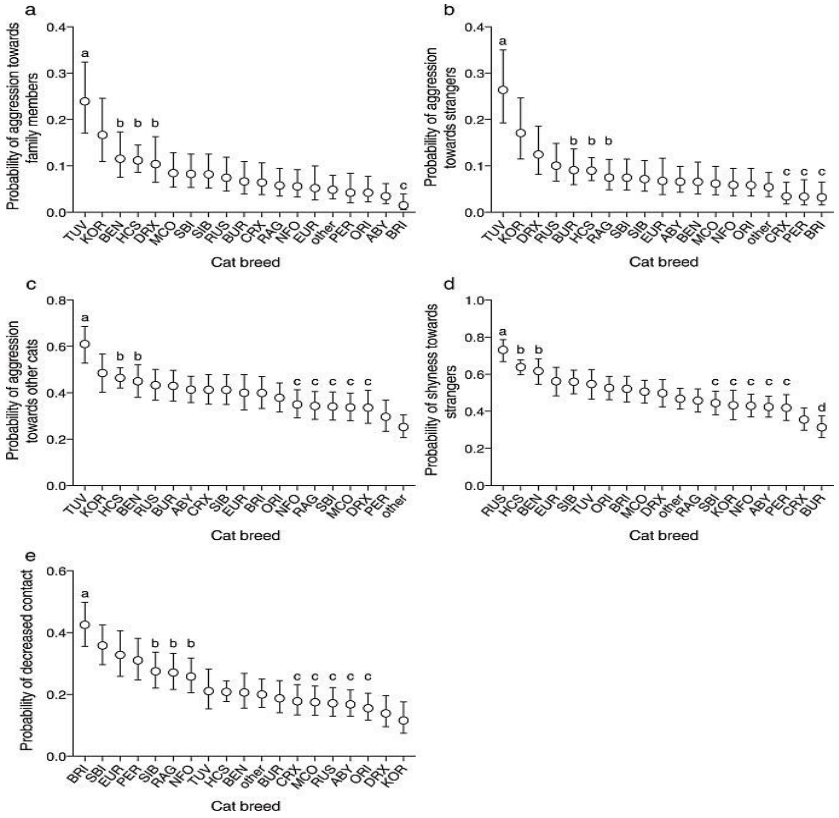

Fig. 3. Breed differences in social behaviour in logistic regression analyses

The letterings indicate groups (false discovery rate corrected P > 0.5 between breeds within the same group) that significantly differ (FDR corrected P < 0.05 between breeds in different groups) from other breed groups. Turkish Van cats were the most aggressive towards family members (a), strangers (b), and other cats (c). Russian Blue cats had the highest probability for shyness towards strangers (d), and British Shorthair cats had the highest probability for decreased contact to people (e). Error bars indicate 95% confidence limits. N = 5726. ABY = Abyssinian, Somali, and Ocicat, BEN = Bengal, BRI = British Shorthair, BUR = Burmese and Burmilla, CRX = Cornish Rex, DRX = Devon Rex, EUR = European Shorthair, HCS = house cat, KOR = Korat, MCO = Maine Coon, NFO = Norwegian Forest Cat, ORI = Balinese, Oriental Longhair, Oriental Shorthair, Seychellois Longhair, Seychellois Shorthair, and Siamese, PER = Persian and Exotic, RAG = Ragdoll, RUS = Russian Blue, SBI = Saint Birman, SIB = Siberian and Neva Masquerade, TUV = Turkish Van and Angora. Odds ratios, their confidence limits, and P-values shown in Supplementary Tables S2–S5 and S7 (11).

One particular case of aggressive behavior in cats is the so-called dysthymia of old cats. Elderly, dysthymic cats show variations in mood, which are manifested by the appearance of unpredictable aggressive phases. The "rolling-

skin syndrome" occurs in this situation and is characterized by the appearance of dorsal horripilation waves that travel through the dorsal region from back to front. Most often, during these episodes, there are frantic tail movements associated with meowing and tail bites, which can lead to self-harm (8). The cat will express aggression towards any moving environmental object. These phases are also characterized by the reduction of the sleep period, which rarely exceeds 6 hours per day. The phases described alternate with the phases during which the cat has normal behavior, the dysthymia of the old cat being a unipolar dysthymia.

From an etiological point of view, it is hypothesized that aging is not the only possible cause of dysthymia. Dysthymia can also be iatrogenic, and it seems that megestrol acetate used to limit or repress the territory marking behavior, can induce such disorders. Meningiomas can also be involved, as well as infections with the virus that causes feline infectious peritonitis (FIP). In some cases, Aujezski virus infection can cause typical, dysthymic behavioral disorders. Hyperthyroidism can also induce and express the clinical picture of dysthymia.

In establishing the diagnosis of old-cats' dysthymia, some criteria must be taken into account: it occurs in cats over 8 years old, the alternation between episodes of irritability and hypervigilance with periods of calm, the phase of irritability characterized by staring at the animal, mydriasis, spontaneous sequences of aggression due to irritation - ears lowered, hair ruffled, tendency to adopt lateral decubitus and externalize its claws, tail movements, accompanied by insomnia - less than six hours of sleep per night, rolling-skin syndrome and the occurrence of tail injuries due to self-harm.

Drug treatment may be recommended and involves, as in dogs, the administration of selegin (3). Selegin-based treatment will be prescribed for at least six months, after which it will stop, but the owner will be asked to report the reappearance of signs, manifestations that could suggest recurrence and impose the resumption of the treatment, many cases a lifelong treatment being necessary.

In conclusion, it can be appreciated that, like involution depression, dysthymia can have organic causes, and one of the diagnostic elements is the etiological one. The evolution of this disorder is dominated by the shortening of the calm periods between two phases of crisis.

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ANATOMICAL ABNORMALITIES IN THE CONFORMATION OF THE LUMBOSACRAL SPINE IN DOG AND CAT

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Summary

Our observations were focused on the lumbosacral region of the spine in dogs and cats in order to identify the abnormalities in the anatomical conformation regarding the number of the lumbar vertebra or other morphological aspects. For this purpose were analyzed the radiographs of the lumbosacral region of 450 canine patients and 232 feline patients of a veterinary clinic. The x-ray exam revealed various pathologies of the spine as the presence of the osteophytes on some vertebrae, the stenosis of the intervertebral foramina consecutive of the inflammatory processes, the mineralization of the intervertebral discs, modified angulation between adjacent vertebra and others and the next anatomical particular aspects of the lumbosacral region: in two canine specimens, a French Bulldog (3 years) and a Boxer (6 years), it was identified a supernumerary lumbar vertebra, the lumbar region containing 8 vertebra; the shortening of the last lumbar vertebra in the same canine patients, but also in other 2 canine patients (a Pekingese of 7 years and a dog of common breed - 10 years) having 7 lumbar vertebra and in a cat of European breed (5 years); the merging of the last lumbar vertebra with the sacrum bone (sacralization) in the same Boxer dog and the same cat. The most common anatomical particular aspect of the lumbosacral spine in domestic carnivores that we found was the presence of the supernumerary vertebra (8LV), but generally associated with the others.

Keywords: dog, cat, supernumerary lumbar vertebra, short lumbar vertebra, sacralization

Generally, the number of the vertebra of the spine regions is constant in mammal species. It is well known that the cervical region is the most constant, almost all the mammals having 7 cervical vertebrae (3), few exception being known. As a rule, all mammals have the same number of vertebrae in their necks regardless of whether they are a giraffe, a mouse, or a human. But both sloths and manatees are exceptions to this rule having abnormal numbers of cervical vertebrae, without apparent problems. Two-toed sloths (*Choloepus didactylus*) have 5-7 neck vertebrae while three-toed sloths (*Bradypus tridactylus*) have 8 or 9 (3, 24, 28). The manatees *(Trichechus)* have 6 cervical vertebra (4). In the rest of the regions, excepting the caudal one, the number of the vertebra can varies from a mammal species to another, but generally is constant from an animal to another inside of a species. In domestic mammals, the largest variation regards the thoracic vertebra in pigs, the number of the vertebra may vary between 14-17. Also, in the sacral region, the pigs can have 4 or 5 vertebra. Another variation can exist in the lumbar region of the horse, some animals having 5 vertebra, instead of 6 (5, 14, 23). This variability is usually in relationship with the breed and is considered normal without affecting the animal health.

The vertebral formula in domestic carnivores includes: 7 cervical vertebrae, 13 thoracic vertebrae, 7 lumbar vertebrae, 3 sacral vertebrae (fused, forming the sacrum bone) and a variable number (20-23) of caudal vertebrae (5, 14). Anyway, there are studies that revealed the most of the particular aspects of the spine target the lumbar or lumbosacral region, especially in dogs, and are considered abnormal. These abnormalities can be clinically insignificant or can conduct to some pathologies of the spine, but not only (6, 9, 11). The main abnormal aspect reported for the dogs was a longer lumbar region, consecutively to the presence of the 8th lumbar vertebra, knowing as the lumbosacral transitional vertebra (LTV). It frequently occurs between the last normal lumbar vertebra and the first normal sacral vertebra. It is considered a congenital anomaly and has morphological characteristics of both lumbar and sacral vertebrae (6, 9). Normal dogs have seven lumbar vertebrae, all separated by intervertebral discs, a complete intervertebral disc between L7 and the sacrum, no contact between the transverse processes of L7 and the sacrum or the ilia, no rotation of L7, sacrum, or pelvis over either their vertical or sagittal axes (6). These normal aspects can be affected by the presence of the LTV, but other abnormalities can appear in the absence of the LTV. For that, we analyzed the radiographs of the lumbosacral spine of some canine and feline patients in order to identify such abnormalities in the conformation of the spine associated or not with LTV.

Materials and methods

The observations were conducted on some dog and cat patients of a veterinary clinic. For our purpose were selected the animals subjected to the x-ray exam of the lumbosacral spine. Totally, the studies involved 450 dogs and 232 cats, both males and females. The animals were brought to the clinic for various pathologies, presenting especially nervous and locomotor symptoms. The patients were clinically examined and then the x-ray exam of the spine was performed using the latero-lateral and ventro-dorsal incidences. The radiographs obtained were analyzed to diagnose the pathology, but also for the anatomical conformation of the lumbar and sacral spine, the number of the vertebra being counted.

Results and discussions

The x-ray exam revealed a lot of pathological processes along of the lumbosacral spine of the patients such as: the presence of the osteophytes on some vertebrae, the stenosis of the intervertebral foramina consecutive to the inflammatory processes, the mineralization of the intervertebral discs, modified angulation between adjacent vertebra and others, but also were identified 5 animals having anatomical abnormalities of this region. In two dogs, a male French Bulldog of 3 years and a male Boxer of 6 years, it was observed that the lumbar spine counts 8 vertebra, not 7, (Fig. 1, Fig. 2, Fig. 3) being identified a supernumerary lumbar vertebra (8LV). In the same animals, the last lumbar vertebra was shorter that the rest. This aspect was observed in the radiographs of other two dogs, a female (10 years) of common breed

(Fig. 4) and a male Pekingese of 7 years (Fig. 5), but the lumbar spine counted 7 vertebrae. The shortening of the last lumbar vertebra was seen also in a cat (5 years) of European breed (Fig. 6). Another abnormal aspect met at the x-ray exam was the merging of the last lumbar vertebra with sacrum bone, or the sacralization. This aspect was found in the same male Boxer dog and the same European cat (Fig. 2, Fig. 7).

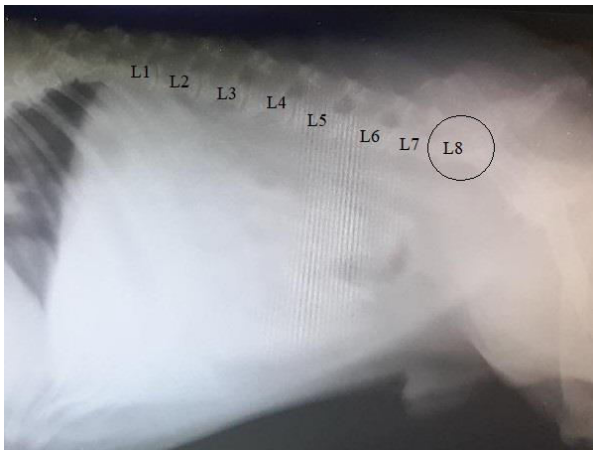

Fig. 1. French Bulldog, Male, 3 years – 8 lumbar vertebrae; the shortening of the last lumbar vertebra (L8); latero-lateral view

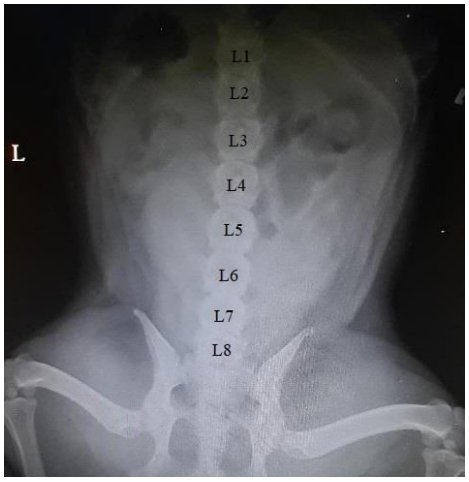

Fig. 2. French Bulldog, Male, 3 years – 8 lumbar vertebrae; the shortening of the last lumbar vertebra (L8); ventro-dorsal view

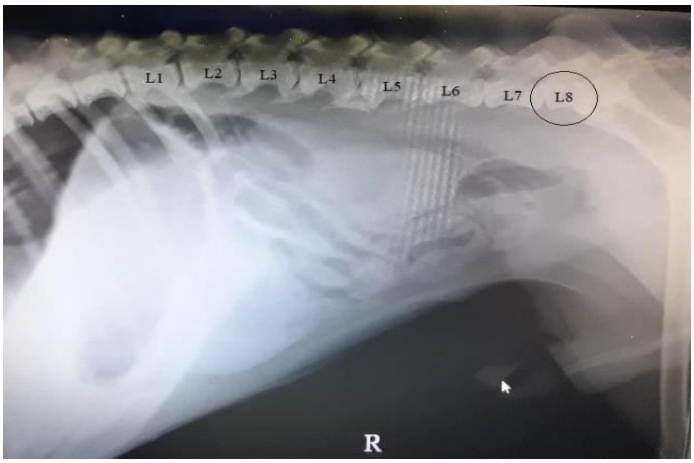

Fig. 3. Boxer, Male, 6 years - 8 lumbar vertebrae; the shortening of the last lumbar vertebra (L8) and the sacralization of 8LV; latero-lateral view

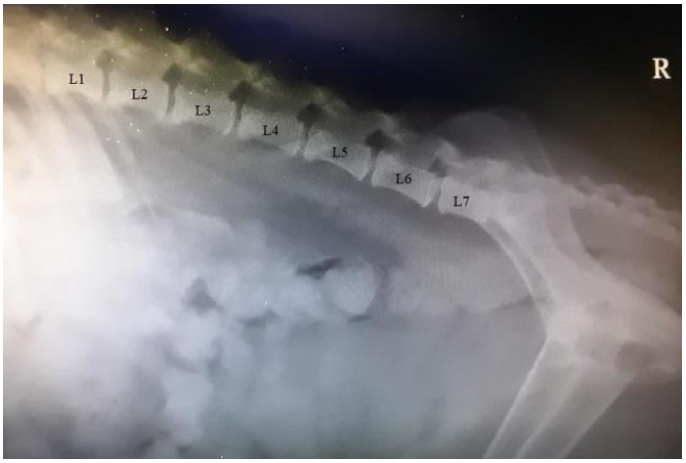

Fig. 4. Common breed dog, Female, 10 years – 7 lumbar vertebrae; the shortening of the last lumbar vertebra (L7); latero-lateral view

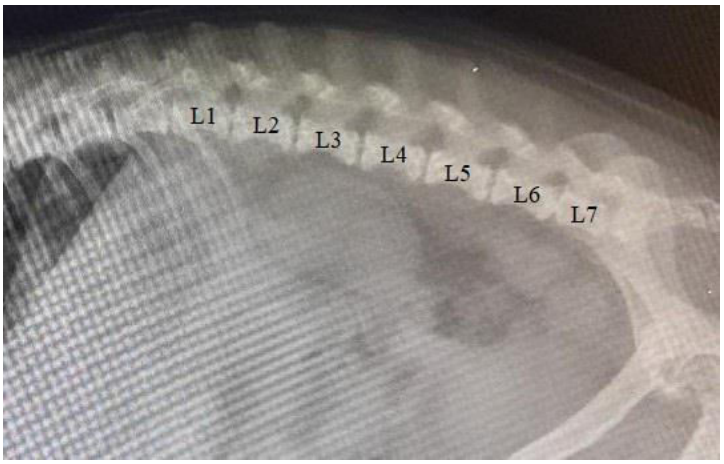

Fig. 5. Metis of Pekingese dog, Male, 7 years – 7 lumbar vertebra; the shortening of the last lumbar vertebra (L7); latero-lateral view

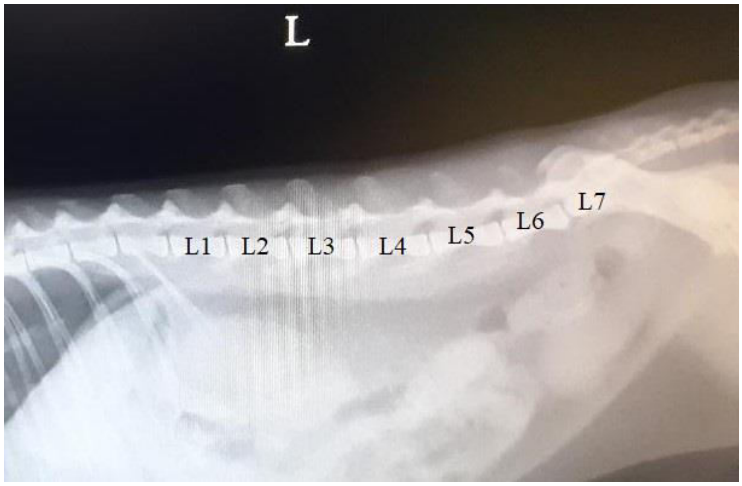

Fig. 6. European cat, 5 years – 7 lumbar vertebrae; the shortening and the sacralization of the last lumbar vertebra (L7); latero-lateral view

The simply presence of the 8LV usually has no clinical importance, and for that it can be considered as an anatomical variation of the lumbar region (16), being known its large incidence. Our observations showed that, generally, the 8LV is shorter, as the last lumbar vertebra when the lumbar region contains 7 vertebrae. The most of the problems were reported when the supernumerary vertebra 8LV is

associated with lumbosacral transitional vertebra (LTV). A lumbosacral transitional vertebra (LTV) is described as an abnormally formed vertebra between the last normal lumbar vertebra and the first normal sacral vertebra (9, 17) or can refers to the forming of an abnormally vertebra resulted by the merging between the last normal lumbar vertebra and the first normal sacral vertebra (1, 6, 11). This aspect is described as a sacralization process, when the last lumbar vertebra fuses with the sacrum bone, or lumbarization, when the first sacral vertebra fuses with the last lumbar vertebra (1). The studies have reported a large prevalence of LTV, from 2.3 to 40.4%, depending on the evaluation criteria and the sample population (11). A high prevalence was reported by Gong et al. (11) in some breeds: Pug (63.6%), Jack Russell Terrier (27.6%), Yorkshire Terrier (19.3%), Chihuahua (17.6%), French Bulldog (17.5%) or Doberman Pinscher (16.7%).

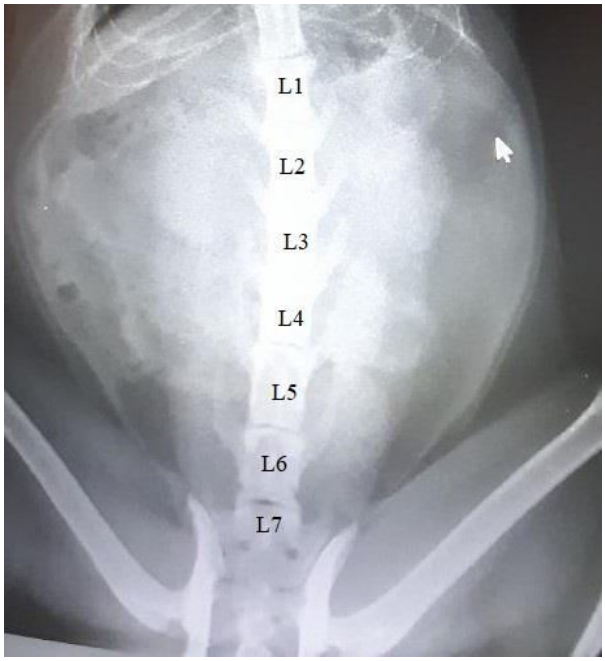

Fig. 7. European cat, 5 years – 7 lumbar vertebrae; the shortening and the sacralization of the last lumbar vertebra (L7); ventro-dorsal view

In LTV, the vertebral bodies demonstrate varying morphology, ranging from broadened transverse process to complete fusion (13). These variations are affected by gender, developmental factors and race. It has been proved that this anomaly is seen to affect males more than females (7). It is also proved that sacralisation is more common in occurrence than lumbarisation - 2:1 (7).

In the morphology of an LTV vary particularly its transverse processes, which may be those of true transverse processes that are not attached to the ilia or sacrum or may be transverse processes that are partially or completely attached to the sacrum and/or the ilia (2, 6, 18, 20, 27). It is mentioned that, the LTV can be symmetrical or asymmetrical. The transverse processes of a symmetric LTV are identical or similarly (9). An LTV is asymmetric when the transverse processes have a different morphology (2, 6, 18, 27). Variations in the shape of the vertebral body are less common (6, 17, 26).

The LTV is considered a congenital anomaly, which occurs in various species of animals and in humans (9) and its presence can be clinically significant. In addition, a hereditary predisposition to LTV has been suggested (6, 8, 18, 19, 20, 21, 25). The condition of the presence of LTV is known to predispose dogs to develop DLSS (degenerative lumbosacral stenosis) in dogs (22) and can lead to premature degeneration of the lumbosacral junction and cauda equina syndrome (CES). This aspect was particularly observed in German Shepherds (9, 10, 19). There was reported that the dogs with an LTV were eight times more likely to develop CES than dogs without an LTV and German Shepherd dogs were eight times more likely to develop CES compared with other breeds. The same studies highlighted that the male dogs were twice as likely to develop CES than females. Dogs with an LTV develop CES 1-2 years earlier than dogs without an LTV (9). Even the number of dogs was reduced, our studies can confirm the predisposition of the male gender to this anomaly, all the dogs identified with 8LV or LTV being males. Also in cats was reported that the lumbosacral transitional vertebrae were significantly more prevalent in cats with lumbosacral stenosis compared with the control feline population and, despite the lumbosacral stenosis is a rare spinal condition in cats, lumbosacral transitional vertebrae can be considered a risk factor for its development (12). In the radiograph of our cat patient we remarked a large degree of sacralization of L7, both transverse processes and the vertebral body being fused with those of the first sacral vertebra (Fig. 7).

Moreover, asymmetric LTV types may lead to pelvic misalignment beyond the vertical axis, causing a unilateral load increase on the hip joint, which results in detrimental development of the hip joint (6, 15, 10).

Conclusions

The anomalies of the lumbosacral spine in carnivores consist especially in the presence of a supernumerary lumbar vertebra (8LV), which is generally shorter than the rest. It is relatively frequent in dogs, and the x-ray exam can be relevant to identify its presence. It affects rather the males and according to the data, can be clinical significant when is associated with LTV, especially the asymmetric type, or with sacralization. LTV may occur also in cats, but in a lower rate, as the presence of a shorter 7th lumbar vertebra.

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STAPHYLOCOCCAL PORTAGE AND THE CORRELATION BETWEEN STAPHYLOCOCCAL SPECIES ISOLATED FROM MILK AND THOSE FROM MILKERS

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Summary

The most commonly isolated bacterium in milk is *Staphylococcus spp*. It is the most common pathogen isolated from both milk and milkers. Our study shows that *Staphylococcus aureus* was isolated from milk and carriers, at the same time coagulase-negative staphylococci were isolated. The samples came from Timis County and were collected for a period of three years (2017 - 2019). Out of a total of 223 milk samples, 42 nasal swabs, 56 samples collected from the milkers hands and 18 samples from the udder sanitation towels, 138 (61.06%) strains of *Staphylococcus spp.* were isolated from milk, respectively 12 (28.57%) from the samples of nasal swabs, 18 (32.14%) from the milkers hands and 2 (11.11%) from the udder sanitation towels. The importance of this research demonstrates that when pulsed field electrophoresis, plasmid typing or other advanced identification techniques are not possible, than the use of any combination of phenotypic characteristics is recommended for epidemiological investigations.

Keywords: *Staphylococcus aureus*, milk, milkers, correlation

Staphylococcus aureus is one of the most commonly isolated bacteria from milk and dairy products, including from veterinarian staff and milkers.

Taking into consideration this fact, the current research aims to isolate and identify the main species of staphylococci from milk and from carrier, including the presence of coagulase-negative staphylococci. The samples were collected from 11 farms in Timiș County, for a period of three years (2017-2019). Thus, 226 milk samples, 42 nasal swabs, 56 samples collected from the hands of the milkers and 18 samples from the udder sanitation towels were processed.

Following laboratory examinations, 170 strains of *Staphylococcus spp*. were isolated and identified, as follows: 138 (61.06%) strains of milk, 12 (28.57%) strains of nasal swab samples, 18 (32.14%) from milkers hands and two (11.11%) from udder sanitation towels. Laboratory determinations were performed using classical, standardized methods, confirming their importance in the current epidemiological investigations, even in the conditions in which various modern methods are available, including: pulsed field electrophoresis, plasmid-typing or other advanced technologies.

In the process of obtaining raw milk there is a major risk that it will become contaminated with numerous microorganisms (7), being known that over a hundred bacterial species colonize the udder.

Although bacteria, fungi, yeasts and possibly some viruses can cause udder infection, the main identified contaminant is bacteria, of which it can be mentioned a large number of species, such as: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, Gram-negative major pathogens: *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia spp*., minor pathogens: coagulase-negative staphylococci (non-aureus staphylococci), *Micrococcus spp.*, *Staphylococcus pseudintermedius*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Streptococcus canis*, *Trueperella pyogenes* and other members of the family *Enterobacteriaceae*. However, the species *Staphylococcus aureus* is considered the main cause of mastitis (15, 16).

This is due to the fact that staphylococci, having a wide range of localization, are present both on the surface of the skin, on some objects and also in natural cavities of humans and animals, as epiphytic and saprophytic bacteria, capable of triggering infections. Thus, we can say that *Staphylococcus aureus* is considered the most important pathogenic germ that produces mastitis, due to its virulence and its special resistance to antimicrobial substances (9, 19).

According to the data cited in scientific literature, in addition to positive coagulase staphylococci, many other species of coagulase-negative staphylococci, known as non-aureus staphylococci, have been isolated (1), which is not surprising, the staphylococcal infection being in a continuous dynamic (2).

Materials and methods

The research was performed on samples taken from 11 farms in Timiş County, located in several geographical areas. A total of 226 milk samples, 42 samples from milkers nostrils, 56 samples from milkers hands and 18 samples from sanitizing towels were collected (Table 1).

Sampling was performed differently, depending on the place of sampling. Milk samples were collected in sterile bottles, while from milkers (hands, nose and sanitation towels) using sterile swabs.

Isolation and identification of species belonging to the genus *Staphylococcus* was performed, according to the method of analysis provided by the standards in force, on selective culture media, identification and confirmation being performed based on the biochemical properties of isolated strains, both by classical and API Staph tests (standardized biochemical system), which allow the classification of strains in species of the genus.

The characteristic colonies, developed on the surface of the selective medium, were subjected to the coagulase test for confirmation, using rabbit plasma. Samples were cultured on trypticase soy agar with 5% sheep blood (TSA), incubated

at 37°C, and examined after 24 and 48 hours. The Baird-Parker medium (BPA) was also used in parallel.

To confirm the nasal passage of staphylococci, samples were usually collected using sterile commercial dry swabs, knowing that there is no difference between dry and wet swabs (25).

The sampling procedure consisted of rubbing the swab in the milkman's nostrils with a few rotating movements. The swabs were then analyzed for staphylococci. The detection method was performed on Giolitti and Cantoni broth and then the confirmation was done using Baird Parker agar by inoculating the culture medium selectively with the specified amount of sample for analysis and incubation of tubes at 37°C for 24 to 48 hours.

Table 1

Origin of milk samples and incidence of staphylococcus strains

Sample area (geographic area of Timis county)	Milk samples / isolated staphylococci	Samples from milkers / incidence of staphylococci				Total samples / total staphylococci
		Nose	Hands	Sanitation towels	Total samples / total isolated staphylococci	
N-V 1	27/14	6/1	8/2	3/1	17/4	44/18
N-V 2	12/9	2/1	3/1	1/0	6/2	18/11
N-V 3	24/18	5/2	7/3	2/0	14/5	38/23
N-V 4	28/16	4/1	6/1	2/1	12/3	40/19
N-V-5	25/19	5/2	6/3	2/0	13/5	38/24
S 1	18/11	3/1	4/1	1/0	8/2	26/13
S 2	23/13	4/1	5/2	2/0	11/3	34/16
S 3	16/10	2/0	3/2	1/0	6/2	22/12
S 4	21/12	4/1	6/1	2/0	12/2	33/14
E 1	19/9	4/1	5/1	1/0	10/2	29/11
E 2	13/7	3/1	3/1	1/0	7/2	20/9
Total	**226/138**	**42/12**	**56/18**	**18/2**	**116/32**	**342/170**

The presumed presence of coagulase-positive staphylococci is indicated by a reduction in potassium tellurite. From the Giolitti and Cantoni broth, the surface of the Baird Parker agar plates was streaked with a sterile loop, after that the inoculated Petri dishes were incubated at 37°C for 24-48 hours, obtaining typical colonies, black or gray, glossy and convex, the atypical colonies having the same size as the typical colonies, but presenting different morphologies.

Confirmation of the colonies was performed by inoculation with a sterile loop and transfer to brain-heart infusion (BHI) broth tubes. After 24 hour incubation at 37°C, 0.3 ml of plasma was added aseptically and coagulation process was followed for 4 - 6 hours, with re-examination after 24 hours.

Results and discussions

Analysis of the obtained data, found that the distribution of staphylococci differs depending on the farm, most strains being isolated from farms located in the northwest of the county (66.17%). Mentioning that, from the same area, staphylococcal strains were isolated also from milkers sanitizing towels.

From a number of 138 staphylococcal strains, isolated from milk, it was found that 73 (52.9%) were represented by *Staphylococcus aureus*, the number of coagulase negative staphylococcal strains being 36 (26.08%).

The incidence of *Staphylococcus aureus* in milk samples was 52.90%, slightly higher compared to the incidence of 46.2% observed by some authors (24), although other authors (11) found that the incidence is variable, being between 10% and 80%, aspect due to both endogenous and exogenous factors.

Analyzing the distribution of isolated strains by species, it was found that among the negative coagulase staphylococci, more frequently isolated from milk, the following species were identified: *Staphylococcus chromogenes* (7.25%), *Staphylococcus xylosus* (2.90%) and *Staphylococcus haemolyticus* (4.35%), the results obtained being slightly different but comparable to those of other authors (22) (Table 2).

Among coagulase-negative staphylococci, another performed study (26), isolated from milk samples, more frequently, the following species: *Staphylococcus sciuri*, *Staphylococcus haemolyticus*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Staphylococcus simulans*.

Table 2

Frequency of *Staphylococcus* species isolated from milk and milkers

Species	Frequency milk		Frequency nasal swab		Frequency hands		Frequency towels	
Studied straines	138		12		18		2	
	No.	%	No.	%	No.	%	No.	%
S. aureus	73	52.90	5	41.66	5	27.77	1	50
S. chromogenes	10	7.25	-	-	2	11.11	-	-
S. xylosus	4	2.90	-	-	1	5.55	-	-
S. haemolyticus	6	4.35	-	-	1	5.55	-	-
S. epidermidis	8	5.79	6	50	4	22.22	1	50
S. intermedius	2	1.45	-	-	3	16.66	-	-
S. hyicus	1	0,72	-	-	-	-	-	-
S. simulans	5	3.62	-	-	-	-	-	-
Biochemically identified strains	109	78.98	11	91.66	16	88.88	2	100
Untyped strains	29	21.02	1	8.34	2	11.11	-	-

Staphylococcus aureus is found on both human skin and mucous membranes and is a common cause of serious infections (18). The most common site of transport is vestibulum nasi (or anterior nostrils), which serves as a reservoir for the spread of the pathogen (20). This bacterium can establish solid interactions with nasal epithelial cells through various proteins (4, 13, 27). Due to this fact, this study found that 41.66% of the strains harvested from the milkers' nostrils were *Staphylococcus aureus*.

This aspect has been mentioned by other authors, who have found that a high percentage of *Staphylococcus aureus* colonizes the milkmen nostrils (3) and is not at all surprising since up to 30% of the human population is asymptomatically and permanently contaminated with nasal *Staphylococcus aureus*, contamination that is influenced by both host characteristics and various environmental factors (17). Other authors state that 50% of the strains isolated from the nostrils belong to the species *Staphylococcus epidermidis*, 90.4% of humans being carriers of *Staphylococcus epidermidis* (12).

Regarding the species isolated from the milkers hands, 27.77% were represented by *Staphylococcus aureus*. A percentage of 61.09 was represented by coagulase negative staphylococci and among them the highest incidence had the following species: *Staphylococcus epidermidis* (22.22%), *Staphylococcus intermedius* (16.66%) and *Staphylococcus chromogenes* (11.11%).

Given the fact that many bacterial and environmental factors, (5, 6) influence the survival of bacteria on different surfaces, allowed the isolation of only two strains from the surface of towels, one being classified as *Staphylococcus aureus* and the other as *Staphylococcus epidermidis*. This aspect shows that the viability of staphylococci on towels surface is relatively small (24 - 48 hours) as shown by other authors (14), although the possibilities of contamination are significant.

Conclusions

The incidence of staphylococci strains belonging to the species *Staphylococcus aureus* in the analyzed samples was significant, respectively 52.90% in milk, 41.66% in the nasal vestibule, 27.77% on milkers hands and 50% on milkers' towels.

An increased frequency of coagulase negative staphylococci, both those from milk and those from staff, has been found.

Species with a variable incidence depending on the origin of the sample have been identified, with *Staphylococcus aureus* strains having a higher incidence, especially in milk samples (52.90%).

The obtained results are similar to those found in other geographical areas (8, 10, 21, 23), the differences, sometimes significant, being a consequence of the influence of the multitude of factors contributing to the occurrence of these infections, that cause significant economic loss, precisely by non-compliance with hygiene rules by staff working in this field.

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CARBOHYDRATES AND STARCH CONTENT IN DOG FOOD

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Summary

Starch is not an essential nutrient for dog food, but it is used as an energy source. For the manufacture of dry food, it is also used as a binder in the extrusion process. The aim of this study was to analyze the starch content of commercial dog food in order to estimate the carbohydrate content. 15 samples of dry food were analyzed, 5 samples from each class: economic (E), basic (B) and premium (P). The average starch content was 35.71% for economy class samples, 32.5% for base class and 24.99% for premium class samples. From the total content of soluble carbohydrates (nitrogen free extract - NFE) it was observed that, for all food classes, starch presented a percentage higher than 50% (E - 60.21%, B - 59.57%, P - 53.45%). Given that most of the starch in dog food comes from cereals, being cheaper, based on the results of this study it can be seen that the percentage of cereals inclusion in the composition of commercial dry dog food is high.

Keywords: soluble carbohydrates, starch, dog food, cereals

Cereals are introduced into dry dog food recipes to provide digestible carbohydrates, at a level of 30-60% of total nutrients and fiber (7, 10). Nitrogen-free extracts (NFE) are made up of carbohydrates, sugars, starch and hemicelluloses. In terms of quantity, the best represented complex carbohydrate in cereals is starch (1, 4). The starch content of cereals varies depending on the variety and their varieties (wheat 48-80.5%, rice 50-81%, corn 49.5-78%, sorghum 55-76.5%, oats 49-73%, barley 47.5-72%), as well as the degree of processing (4).

Dry pet food must contain starch, without it being impossible to expand the croquettes during extrusion process. During this process, the ingredients are subjected to a high temperature, above 150°C, which leads to an increase in the size of the starch granules, improving its digestibility and palatability of the croquettes (4). Differences in the digestibility of starch are induced by a number of factors, such as the type of cereals, the starch-protein interaction, the physical shape of the starch granules, the type of starch, digestive inhibitors, processing and particle size resulting from processing (3).

The particle size of the cereals influences the gelatinization of the starch during extrusion, the digestibility of the nutrients in the final dry food and the level of short-chain fatty acids in the faeces, only in the case of some cereals. Research in this area has shown that rice, as a source of starch, has a higher digestibility than

other cereals, regardless of particle size. Instead, maize and sorghum must be ground very finely to achieve levels of gelatinization and digestion comparable to those of rice (1).

The aim of this study was to analyze the content of nitrogen-free extract (NFE) and the starch in dry dog food in order to estimate the proportion of carbohydrates in the composition of commercial diets. For the study, five diets from each type of economy class, basic and premium, purchased from Romania, were randomly selected. The samples taken in the study were processed in the Animal Nutrition - Analysis Laboratory of the Faculty of Veterinary Medicine from Timisoara.

Materials and methods

This study aimed to analyze commercial diets for dogs, in terms of content in nitrogen-free extract (NFE) and starch. The study was conducted between February 2019 - July 2020 and were used, for analysis, 5 brands from each category of diets: economic, basic and premium. A total of 15 commercial diets for dogs were examined (Fig. 1.). Diets for adult dogs with moderate physical activity have been selected, which is the most marketed category of dog food.

Fig. 1. Commercial food samples for dogs

The commercial diets were purchased from pet-shops or veterinary offices and were analyzed within the Laboratory of physico-chemical feed analysis, of the Animal Nutrition and Agronomy discipline, Faculty of Veterinary Medicine, Timisoara. In order to maintain the anonymity of the producers, the diets were divided into the 3 main groups: economic (E), basic (B) and premium (P) and numbering, within each category, from 1 to 5.

Prior to processing, commercial food samples were prepared by grinding, so that the particle size was as small as possible, in order to facilitate the penetration of infrared rays and obtain the most accurate results (Fig. 1). The samples were analyzed in duplicate, in order to obtain the most accurate results, and the values reported as results consisted in the arithmetic mean of the values obtained for each sample.

The Bruker optics Matrix I NIR analyzer, which uses infrared light (Fig. 2), was used to determine nitrogen-free extract and starch in order to estimate the cereal content of commercial dog diets. Near InfraRed spectroscopy is a method of analysis that uses the NIR region of the electromagnetic spectrum (800 – 2.500 nm). It measures the absorption of light from the sample in the NIR region at different wavelengths. Samples can be analyzed non-destructively in seconds for major constituents such as moisture, fat, protein, fiber and ash, as well as other specific parameters such as starch, amino acids and others. Complete Bruker Optics calibration packages for feed and ingredients are developed in accordance with ISO 12099 (14, 15). Bruker optics Matrix I NIR analyzer work with OPUS software that ensures quality data processing.

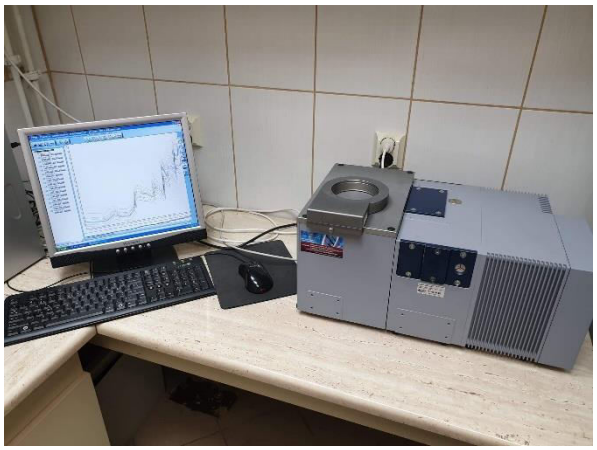


Fig. 2. NIR infrared analyzer

The obtained results were statistically analyzed using Excel Data Analysis. Statistical correlations and descriptive statistics were made and statistical datas were expressed through tables and diagrams.

Results and discussions

In the NIR analysis of the studied commercial diets, according to the classification of economic (E), basic (B) and premium (P) categories, the following results were obtained, presented in Table 1. The results were quantified in percentage (%) reported to 100 g of sample:

Table 1

Analysis of NFE and starch content in the studied diets

Diet	NFE %	Starch %
E1*	64.01	32.56
E2*	60.51	39.11
E3*	63.86	43.15
E4*	57.58	36.03
E5*	50.59	27.73
B1**	49.69	31.18
B2**	54.68	33.24
B3**	60.49	37.1
B4**	54.65	35.18
B5**	53.26	25.84
P1***	42.7	18.93
P2***	41.4	9.72
P3***	46.74	28.97
P4***	48.1	34.03
P5***	54.83	33.31

*E 1-5 – Economic class, samples from 1 to 5;
**B 1-5 – Basic class, samples from 1 to 5;
***P 1-5 – Premium class, samples from 1 to 5

At the analysis of soluble carbohydrates (nitrogen-free extract - NFE%) the infrared reading curves (Fig. 3.) identified the following values: for economic diets the values varied between 50.59% and 64.01%, the average value being 59.31 %, for basic food values were obtained between 49.69% and 60.49%, with an average value of 54.55%, and for premium category diets the variations were between 41.4% and 54.83 %, with an average of 46.75%. From the analysis of values, it can be seen that the highest percentage of NFE was obtained in the economy class, and the lowest percentage of NFE - in diets of the premium class.

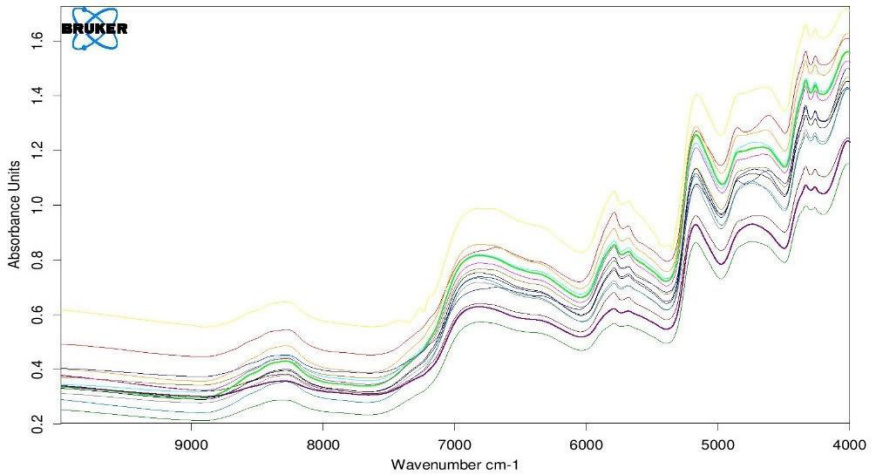
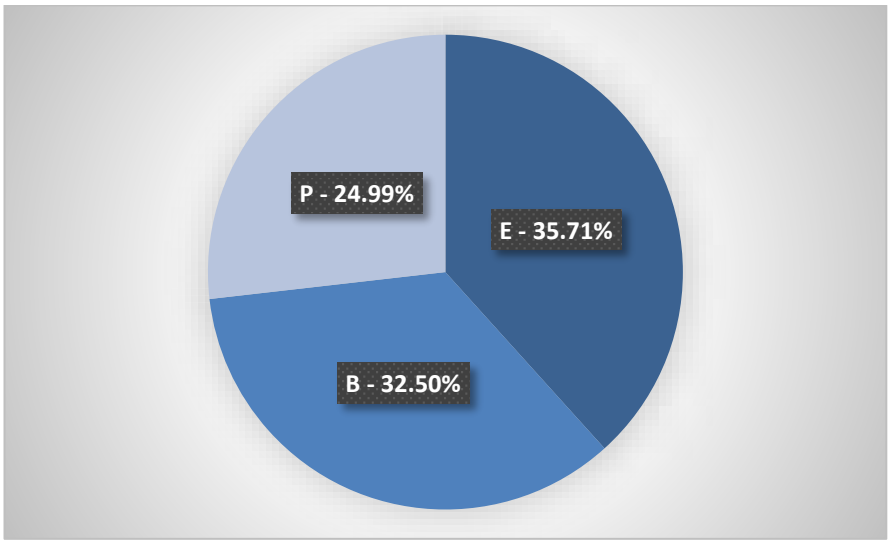


Fig. 3. Infrared analysis of the commercial diets studied

The starch content varied between the categories of commercial diets. For the diets of the economic category, the starch values showed variations between 27.73% and 43.15%. The diets in the basic category showed a starch content between 25.84% and 37.10%. The commercial food of the premium type showed lower values for starch, compared to the other two categories of diets studied, between 9.72% and 34.03%. In the premium category, a special situation was encountered in two of the five diets analyzed in this class, by the fact that the manufacturer stated on the package that the product does not contain cereals, but in the infrared analysis (Fig. 3) a content of 9.72% and 18.93%, respectively was obtained. The data obtained for all classes of diets could not be compared with the producers values, as not all food brands labeled the starch content.

By comparing the two elements analyzed, a direct and positive correlation was observed between the values obtained for NFE and those identified for starch, for each commercial food class investigated. In the economy class, the highest values were obtained for NFE (average - 59.31%) and also for starch (average - 35.71%). The class with the lowest values for the two parameters studied was represented by the premium category, where the average value for NFE was 46.75%, and for starch 24.99%. For the basic class, the values obtained by analysis were in the middle, by comparing the three classes of commercial food (NFE - 54.55%, starch - 32.5%) (Fig. 4).



*E – economic; B – basic; P – premium

Fig. 4. The average values of the starch content in the three types of diets studied

Calculating the percentage of starch content from the soluble carbohydrates (NFE) it was observed that, for all food classes, the percentage was higher than 50%. Thus, in the economy class, the percentage of starch of the whole NFE was 60.21%, for the base class, the percentage was close to the previous one, with a value of 59.57%, and for the premium class, the starch content of the whole NFE was of 53.45%.

The starch content is important in assessing the addition of cereals in commercial diets, starch being the majority constituent of these vegetable ingredients. Thus, the richer the starch content of a commercial diet is, the more suspicious the inclusion of an increased percentage of cereals in the manufacturing recipe. The high content of starch, correlated with an increased percentage of cellulose in a commercial dog food, can predispose animals to various digestive disorders, which implicitly alter the absorption of other nutrients, leading to qualitative malnutrition with special effects on the pet health.

In the literature, the interest in studying the proportion of carbohydrates in dogs' diets is growing. Thus, Corsato Alvarenga and Aldrich from the USA, in 2020, conducted a study on the starch characterization of commercial extruded dry pet foods, and concluded that the regression analysis of NFE and total (calculated) starch can be a useful tool to assess the total starch content of commercial dog food (5).

Sandri et al. (13) in 2019, tested the digestibility of various sources of starch

included in home-prepared diets, as well as the effect of starch fractions on the canine microbiome. They noticed that the starch digestion rate was much higher in the diet containing rice (7.2% / min) and lower in the potato starch diet (3.8% / min), however, the starch fractions do not seem to have an effect on the bacterial population in the dogs studied (13).

Regarding the starch content and digestibility of soluble carbohydrates, Calabro et al. (2) tested the degree of digestibility of these compounds in commercial diets for dogs and how their presence in food affects the intestinal health of animals. They emphasized that the control of intestinal gas production should be an important objective in the selection of feed ingredients, including the types of carbohydrates, including starch fractions, in order to avoid undesirable effects such as flatulence and soft faeces (2).

The study by Murray et al. (10), showed that the extrusion of cereal grains and potatoes at both low and high temperatures changes the concentrations of fast digestible starch, slow digestible starch and resistant starch, compared to raw forms of them. In addition, an ileal microbial population capable of fermenting starch from various sources, fractions and processed forms of it is located in the small intestine of dogs and can contribute to the general use of starch. This bacterial population is significant in determining the starch fractions that can be fermented bringing benefits to the general health of the colon. Understanding these differences in using between different sources of starch and their processed forms will allow the precise use of cereal grains in pet diets (10).

Cereals are included in dog food because they are an important source of energy, participating in ensuring the level of protein and other nutrients, such as thiamine and niacin. The most important cereals used as an energy source in pet food are maize, rice, wheat, barley and sorghum (16). They are used as such (the whole grain, consisting of the shell, endosperm and germs) or only part of them. For example, whole grains contain 75% starch and other digestible carbohydrates, 6-10% protein, 4-5% fat and 7% fiber. Cereal processing generates by-products that can concentrate certain ingredients, such as protein, fiber, starch or oil, and which, when added to pet food, are a source of highly digestible nutrients (7). Providing moderately fermentable fiber, such as rice bran, has additional beneficial effects on the digestive tract (16).

As cereals are usually introduced in the form of flour, they will bring significant starch intake, to which specific protein is added, as well as moderate or low amounts of fiber and fat. Wheat contains a higher level of protein than other grains. There are studies that have shown that gluten - wheat protein - is actually a combination of two proteins (glutenin and gliadin) and is recognized as the cause of chronic enteropathy in the Irish Setter (12) and, more recently, in the Border Terrier (8, 9), similar to celiac disease described in humans, in gluten intolerance.

The majority of starch is digested in the small intestine, but a fraction of it, along with degradation products, is resistant to enzymatic hydrolysis and passes into the large intestine. Here, this digestion-resistant starch (DRS) induces changes in

the microenvironment, affecting the local population of bacteria and, consequently, fermentation, fecal pH and butyrate concentration, which increases. Short-chain fatty acids derived from DRS fermentation are a major substrate for the energy metabolism of colonocytes, and butyrate acts as a growth factor for epithelium (6, 11). Thus, diets with a high DRS content are especially recommended for senior dogs (11). One of the methods of including DRS in diets is the use of coarsely ground cereals, especially maize and sorghum (1). However, studies on large breeds of dogs (German Shepherd and Giant Schnauzer) show that supplementation with more than 2.5% of food with DRS leads to gastrointestinal disorders, with a concentration of 7.4%, dogs presented signs of acute colitis, because of an excessive fermentation in the large intestine, with bloody diarrhea, as opposed to small breeds (Miniature Schnauzer and Pudel) that tolerate high levels of DRS (6).

Starch is not an essential nutrient for dog nutrition, but it can affect health in different ways, depending on how it is included in the diet, the type and degree of its processing.

The analysis of commercial diets for dogs would be very important, as they may have a different nutritional value compared to the nutritional value from the label. Without correct information on the nutrient content of dog food, it is impossible to feed dogs properly. Over time, the result of feeding on common commercial diets, not individualized according to the needs of each animal, may consist in the development of certain intolerances to some nutrients, or even the result of various pathologies of nutrition and metabolism.

Conclusions

The starch content showed high values in all three categories of commercial food investigated.

For the economy class, the highest values of NFE and starch were identified.

The percentage of starch from the whole NFE was higher than 50% for all types of commercial food studied.

Starch is a compound present in high amounts in commercial dog food.

Based on the high content of NFE and starch, it can be estimated that the degree of inclusion of cereals in the composition of dry dog food is high.

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MILK PHYSICOCHEMICAL PARAMETERS: EFFICIENCY OF LACTOSCAN

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Summary

This research was conducted to study the physicochemical parameters of samples of raw bovine milk by conventional methods and using of lactoscanSP. A total of 113 samples of raw milk were collected from the dairy between from April to November 2020. Results showed a variation in pH, density, lactose, protein, fat, solid not fat (SNF) but generally comparable to the national and international requirements standard. However, there is a significant difference between parameters obtained by lactoscan and that of classical methods (PH, Fat, Density and Conductivity) $p<0.0001$).

Key words: raw Milk, LactoscanSP, quality, physicochemical parameters

Algeria is a largest consumer of milk in the Maghreb, with an average consumption need of 3.8 billion liters per year. However, the level of milk collection remains low; nearly 25% of national production, estimated at 2.5 billion liters per year for a herd of approximately one million dairy cows (21).

Attention is paid in particular to raw milk, being an unprocessed product, not undergoing any treatment and therefore retains all its natural properties. Faced with consumer who demand which is increasingly asking for innovative and good quality products. Indeed, the difficulty lies in this last concept which remains very subjective and has different definitions at each level of the sector. The manufacturer demands a raw material with a high processing yield, while the consumer wants a product without pathogenic risk and with satisfactory organoleptic qualities (33).

The physicochemical and bacteriological quality of the milk remains irregular because of several factors, such as the feeding of the cattle, the lack of hygiene, the breed, and the season which constitute preponderant factors of the poor quality of milk (23). However, the production of cow's milk often comes up against the problem of quality management which penalizes both producers and processors. Hygiene conditions at farm level, the interruption of the cold chain along the production circuit until the milk arrives at the dairy, include so many sources of contamination to be controlled in order to preserve hygienic quality milk (12).

Some methods used to determine milk physicochemical quality as the determination of fat, protein, lactose, minerals, etc. have been improved. Automatic devices, including the LactoscanSP allowing several parameters in record time are largely used.

In this sense, we are interested in pH, Fat, conductivity, and density which can be important indicators. The principle of this study consists to evaluating the physicochemical quality of raw cow's milk by conventional methods vs use of lactoscanSP (Ultrasonic Milkanalyser) in the city of Tiaret (western Algeria).

Materials and methods

The region of Tiaret is located in the high plateau of Algeria, at an altitude of 1086 m (350°15' NR, 10°26' E); a semi-arid area characterized by cold and humid winter and hot and dry summer. The work was conducted in a state dairy and Laboratory of Hygiene and Animal Pathology, Institute of Veterinary Sciences, University of Tiaret, Algeria.

A total of 113 samples of raw milk were collected from the dairy between from April to November 2020.

Cow's milk arrives at the factory in bulk in insulated tank trucks. Upon receipt, milk temperature was measured immediately using a thermometer; if temperature is high, the milk was rejected. Thereafter, rapid tests were performed: acidity, density, fat, antibiotic test, temperature, and conductivity. Depending on the result, all considered milk may be refused.

1- Physicochemical analysis

1. pH measurement: pH was measured using a pH-meter (HANA instrument) (22).
2. Acidity: was evaluated by titration with 1/9N NaOH in the presence of phenolphthalein, acidity of milk was expressed in degrees Dornic (°D) (2).
3. Density: Milk density was determined with a lactodensimeter (Funk Gerber). It is reduced to 20°C by the following formula: corrected density = read density + 0.2.
4. Electrical conductivity was measured using a conductivity meter (HI 5222, Hanna Instruments), after each measure, the probe was washed with distilled water and cleaned.
5. Fat: determined by acid-butyrometric method of Gerber (3) that consists in attack of milk with sulfuric acid and separation by centrifugation in presence of isoamyl alcohol of the released fat (4).
6. Antibiotic test: was carried out using Betastar® combo, a rapid (5 minute) lateral flow assay for the visual detection of beta-lactam and tetracycline antibiotic residues in raw milk (26).

Then, Lactoscan SP (Milcotronic, Bulgaria) was used to compare some milk parameters as shown in Table 1.

The averages of measurements (pH, density, conductivity, and fat) were compared by means of unequal variance Welch test (32) at confidence interval 95%.

Table 1

Lactoscan Measuring range

Physicochemical parameters	Measuring range (manufacturer)
pH	0 à 14
Density	1015 à 1040 kg/m^3
Conductivity	3 à 14 mS/cm
Fat	0.01 à 25%
Solid no fat	3 à 15 %
Protein	2 % à 7 %
Lactose	0.01% à 6 %
Water content	0 % à 70 %
Freezing point	-0.4° C à 0.7°C
Salts	0.4% à 1.5%

Results and discussions

Physicochemical comparative parameters

The results of physicochemical parameters analysis are shown in Table 2.

Table 2

Physicochemical qualities

Parameters	Max	Min	Mean	SD	Standards	Satisfactory samples (%)
pH[1]	6.87	6.3	6.45*	0.16	6.6-6.8 (24)	46
pH[2]	6.98	6.2	6.61*	0.17		71
Density (kg/l)[1]	1.032	1.023	1.020	0.093	1.030-1.032 (24)	89
Density[3]	1.029	1.027	1.029	0.006		30
Freezing point (°C)	-0.48	-0.606	-0.550	0.024	≤-0.520 (31)	82.3
Conductivity (mS/cm)[1]	5.86	4.85	5.53*	0.19	4-5.5 (24)	68
Conductivity[4]	6	4.4	5.28*	0.39		84
Fat (g/kg)[1]	49.8	22.5	34.57	0.52	34-45 (36)	71
Fat[5]	41	23	34.30	0.39		66
Protein (g/kg)[1]	51.8	27.2	31.87	0.25	≥29 (36	99
Lactose (g/ kg)[1]	51.9	42.8	47.39	0.19	40–50 (36)	100
Solid no fat (g/l)[1]	94.3	75.1	86.31	0.36	≥85 (36)	70

1: By Lactoscan; 2: By pHmeter; 3: By Lactodensimeter, 4: By Conductimeter; 5: By Gerber

*: significant difference between lines

1- Average pH is estimated at 6.45 and 6.61 with Lactoscan and Ph meter respectively, with no significant difference, in accordance with (6.55) reported by Bousbia et al. (10). However, between 46 % and 71 % samples was moderately acid depending on test (Lactoscan and pH meter respectively).

This value is lower than several values reported in different regions in Algeria: 6.62 (1), 6.54 Benlahcen et al. (9), 6.64 Hamiroune et al. (17), and 6.68 (25). Another study in Tunisia reported a lower value of 6.26 (34).

2- The acidity was acceptable (17.98°D), in accordance with results reported by Aggad et al. (5) in the same region. Acidity can be an indicator of milk quality at the time of delivery because allows to appreciate the amount of acid produced by bacteria or possible fraud (18).

pH and acidity depend on the content of casein, mineral salts and ions, hygienic conditions during the deals with the total microbial flora and its metabolic activity and milk handling (28).

The acidity can be natural due to the stage of lactation, the casein content, mineral salts, and ion content, or it may be due to hygienic conditions during the milking, the total microbial flora and its metabolic activity (29).

3- The density (1.02) calculated by Lactoscan is lower to several values reported in Algeria varying from 1.028 to 1.036: 1.036 (9); 1.029 (27) but also in other countries: Tunisia 1.028 (34); Turkey 1.027 (37); Togo 1.036 (35) and Senegal 1.029 (18). However, when calculated by Lactodensimeter (1.029), it tends to be close to these results.

The density is directly related to dry matter, fat, temperature, and diet of the animal (28).

4- Average milk conductivity was 5.53 mS/cm higher than that reported by Bousbia et al. *(*10) (4.49) but in accordance with standard 4 to 5.5 (at 25°C) (24).

In our study, 13% of cows are infected with subclinical mastitis and twenty percent of them have a risk of developing the disease. The conductivity can be used to detect subclinical mastitis (30).

Hamann and Zecconi (16) reported milk conductivity varies considerably between breeds, individuals of the same breed, depending on diet, lactation stage, milk temperature, material content fat, interval length between two milkings and herd.

5- Fat content ranged from 22.5 to 49.8 g/kg with an average of 34.57±0.52. Results are in range of value reported in Algeria: 26.67 (9), 37.20 (10), Hamiroune et al. (17) in 2014 36.6, 33.3 (28). Algerian norms are 34 g/l while the AFNOR norms are situated between 34 to 36 g/l. Our results are higher than those reported in other countries: Morocco 31.5 (22) and Tunisia 32.5 (34); Togo 31.6 (35) but lower than in Senegal 44.97 (18).

Fats value varies with breed, milking rank, season, and nutritional supply (9, 11).

6- Antibiotic residues: 6% of samples were positive. National and international regulations specify milk must not contain antibiotic residues. This rate

is lower than the one (11.97%) reported in Algeria by Baazize-Ammi et al. (8) but also (14% and 56%) by Aoues et al. (6).

7- Protein content was high 31.37±0.25 variable compared with values reported by Bousbia et al. (10) (29.42); Lounis and Harfouche (25) (24.64). Our results are in interval of value reported in different region of Algeria 29.2 (1), 32.8 (28). But it is lower in comparison with other countries like Tunisia 33.1 (19); Togo 33.4 (35) and Senegal 35.1 (18). The protein level is in relation with the race, the udder health, the lactation, the season and the number of layouts (7, 11).

8- Lactose content varies from 42.8 to 51.9 with a mean of 47.39 g/l. This value is lower than the values reported by Hamiroune et al. - 6.35 (17) and Louni et al. - 59.96 (25). However, it is higher than other values recorded by Bousbia et al. - 44.17 (10) and Matallah et al. - 35.9 (28). It's also higher than reported in Morocco 43.5 (22), Tunisia 44.7 (19) and, Senegal 45.7 g/l (18).

The lactose concentration of milk could be used as an indicator of mastitis (15).

8- Solid not fat value is 86.31±0.36 g/l. This value is lower than reported in Algeria 11.4 g/l (17), 11.26 and in other countries like France 90 (13). But it's higher than those reported in Tunisia - 84.7 g/l (19) and in Turkey - 84.2 g/l (37).

9- Freezing point average value was of -0.55°C different with a previous value (-0.38) reported in Algeria (17) this parameter allows assessment of quantity of water possibly added to milk. A wetting of 1% leads to an increase in the freezing point of around 0.0055°C (14).

In comparison with the physicochemical parameters obtained by lactoscanSP, the results obtained from this study show a variability average in pH; conductivity and density.

Conclusions

The study carried out during the winter period showed that the quality of the milk was generally above the standard. However several factors influence quality of milk such as breed, season, the feeding of the cattle.

Using of lactoscan in dairy play a crucial role because it allowing several parameters in record time without addition of chemical reagents that have altered the nutritional composition and the loss of some quantity of milk. All the results from the LACTOSCAN milk analyzers' analysis are based on a direct measurement of the parameters, which means confidence in results.

Therefore, lactoscanSP facilitates the task of quality control and repression of fraud by measuring addition of water and freezing point. It can also be used as a way for early detection of subclinical mastitis (by multi analysis of: pH, lactose, conductivity). It is providing innovative solutions in the field of milk analyzing and milk testing.

More attention should be given to milk hygiene as well as the determination and control of critical points at farm level.

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CLINICAL AND ANATOMOPATHOLOGICAL STUDIES ON VIRAL ARTHRITIS IN A BROILER FLOCK

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Summary

Viral arthritis is a term used to describe inflammation affecting leg joints and/or tendons in poultry species that is attributable to reovirus infection. Researches has been made on 10 000 broilers from COBB 500 hybrid. The birds were monitored through clinical and anatomopathological exams (macroscopic and microscopic examination) done weekly on the entire growth period, and also until the proper age and weight of slaughter. Sick broilers presented difficult walking, lameness, uni- and bilateral arthritis in tibio-tarsometatarsal joint. The frequency of this syndrome was ranged between 9.09% in cadavers of 19 days of age and 12.50% in cadavers aged between 26 and 28 days. In sections, by HEA staining method, were observed lesions that point a damage of the bone tissue structure. Thus, histopathological lessions encountered are: periosteal congestion and periosteal oedema, incomplete osteogenesis, coagulative necrosis, heterophil accumulation and perivascular infiltration. Researches made in the monitored broiler flock completed and contured clinical and anatomopathological diagnoses for viral tenosynovitis arthritis.

Keywords: reovirus infection, inflammation of leg joints, gastrocnemius tendon rupture

Avian reovirus infection is an infectious contagious disease, produced by reovirus, met in chicken and turkey broilers, with clinical signs of several pathological entities, in which are, more frequent: tenosynovitis arthritis syndrome and malabsorption syndrome (1, 8, 10).

Viral tenosynovitis arthritis is a disease of broilers, with economical importance, that can be caused by different serotypes and pathotypes of avian reoviruses. Viral arthritis is a term used to describe inflammation affecting leg joints and/or tendons in poultry species that is attributable to reovirus infection (11, 12, 15).

Olson et al. in 1957, described a naturally occurring synovitis in chickens from which they were able to isolate an agent serologically unrelated to either *Mycoplasma gallisepticum* or *Mycoplasma synoviae*. This agent, later named the "viral arthritis agent" by Olson and Kerr, eventually was identified as a reovirus by Walker et al. in 1972 (10, 11, 14, 17).

In broilers, economic losses are frequently associated with an increased mortality rate, a general decrease of performances, including unfulfilled body condition score, poor feed conversion, unequal growth rates, slaughterhouse forfeitures and an improper capitalization on the market of those affected birds (4, 5, 6, 13, 16).

Researches had the aim to complete existent data from literature regarding anatomopathological and clinical diagnoses.

Materials and methods

Researches has been made on 10 000 broilers from COBB 500 hybrid, soil grown chicken. The birds were monitored through clinical and anatomopathological exams (macroscopic and microscopic examination) done twice a week on the entire growth period, and also until the proper age and weight of slaughter.

From broilers that had macroscopic lesions were preserved samples (tibiotarsal joints, bones) for microscopic exam processed by classical method and the staining methods used involved Hematoxylin & Eosin (H&E) and Methylene Blue protocols.

The diagnosis of reovirus infection was confirmed by RT-PCR and ELISA test.

Results and discussions

Specific symptoms of tenosynovitis arthritis syndrome appeared in broilers after the age of three weeks. Sick broilers presented difficult walking, lameness, uni- and bilateral arthritis in tibio-tarsometatarsal joint.

Joints have been increased in volume, fluctuating and painful (Fig. 1, 2). Due to this reasons broilers refused movement for food and water intake, thus appearing progressive weight loss.

Fig. 1. Bilateral arthritis in tibio-tarso-metatarsal joint

Fig. 2. Bilateral arthritis

After the age of four weeks, in some broilers appeared the rupture of gastrocnemius tendon, followed by impossibility of movement and doing the extension of affected limb. Usually, these alteration was unilateral (Fig. 3).

Fig. 3. Gastrocnemius tendon rupture

Tenosynovitis arthritis syndrome started in the monitored flock later than the malabsorption syndrome, being absent in cadavers dissected at age of 6, 12, 14, 21, 33 and 41 days. The frequency of this syndrome was ranged between 9.09% in cadavers of 19 days of age and 12.50% in cadavers aged between 26 and 28 days (Fig. 4).

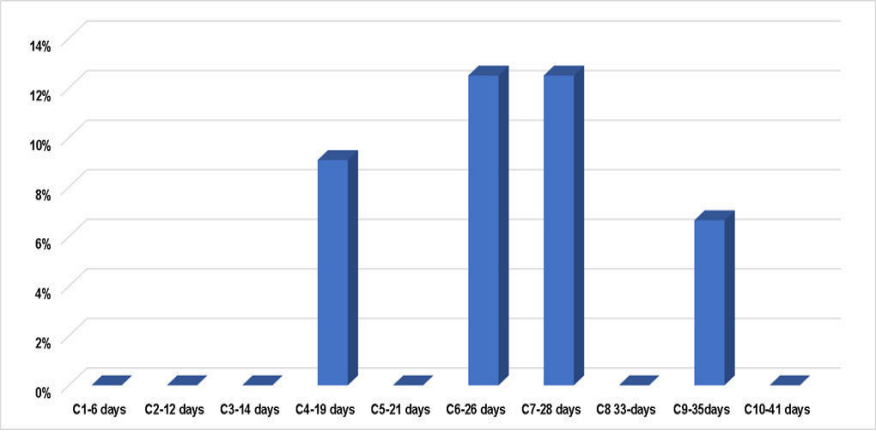

Fig. 4. Frequency of arthritis-tenosynovitis

In broiler cadavers that was reporded this syndrome were highlighted several macroscopic lesions as: serous and fibrinous periarthritis, fibrinous arthritis and fibrin deposits in metatarsal extensors hoods, oedema of digital flexors hoods and rupture of gastrocnemius muscles tendons, this can often be perceived as a greenish discoloration of the skin due to extravasation of blood (Fig. 5, 6, 7).

Fig. 5. Arthritis-tenosynovitis - 28 days old broiler chickens

Fig. 6. Arthritis-tenosynovitis - 21 days old broiler chickens

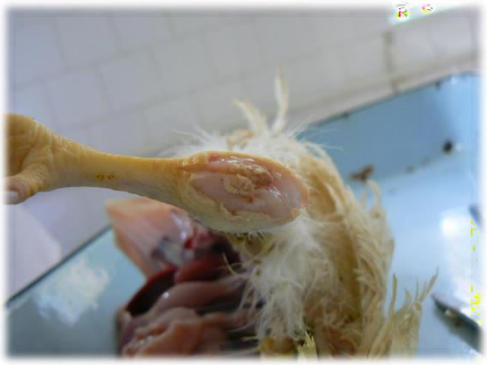

Fig. 7. Arthritis-tenosynovitis - 30 days old broiler chickens

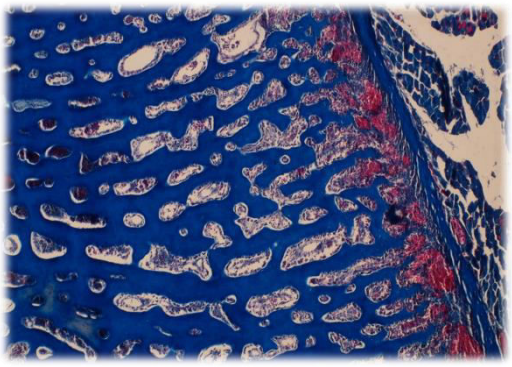

Fig. 8. Bone - histological section, col. HEA, ob x10

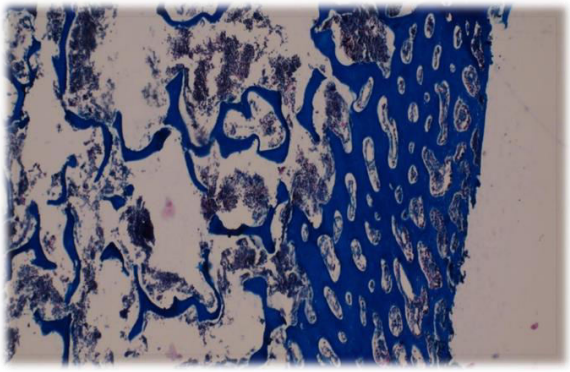

Fig. 9. Bone - Histological section, col. HEA, ob x10

In sections, by HEA staining method, were observed lesions that point a damage of the bone tissue structure. Thus, histopathological lesions encountered are: periosteal congestion and periosteal oedema, incomplete osteogenesis, coagulative necrosis, heterophil accumulation and perivascular infiltration (Fig. 8, 9).

There are also hypertrophy and hyperplasia of synovial cells, an infiltration of lymphocytes and macrophages, and also a proliferation of reticular cells.

Tenosynovitis arthritis syndrome evolved together with malabsorbtion syndrome, an association of those two syndroms being rarely reported in our country. Researches made in the monitored broiler flock completed and contoured clinical and anatomopathological diagnoses for viral tenosynovitis arthritis (2, 3, 7).

Conclusions

Tenosynovitis arthritis syndrome appeared after the age of three weeks, being initally revealed by a difficult walk and lameness, and furthermore by arthritis, tenosynovitis and rupture of gastrocnemius tendon.

Featured anatomopathological lessions were represented by periarthritis, fibrinous arthritis and rupture of gastrocnemius muscles tendons.

The lesions of the long bone denote incomplete osteogenesis and a late onset of the ossification process.

Researches made in the monitored broiler flock completed and contoured clinical and anatomopathological diagnoses for viral tenosynovitis arthritis.

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DETECTION OF ANTIBIOTIC RESIDUES IN RED MEAT

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Summary

The study was carried out to detect the antibiotic residues in raw meat sold for human consumption. A number of 48 meat samples were analyzed (30 pork, 16 beef, 2 mutton), using a qualitative rapid test – Mera Test, based on microbiological method. Of the total samples after three hours of incubation at 64°C, 24 (50%) samples were positive. After an extension of the incubation time by 6 hours, the following results were obtained: 14 samples were positive (29.2%), 6 (12.5%) samples were dubious and 28 (58.3%) were negative. The high proportion of positive samples in the rapid microbiological test for the detection of antibiotic residues in meat is a concern for public health. Further quantitative dosing studies of meat antimicrobial residues are needed to clarify these issues.

Keywords: antibiotic residues, meat, microbiological rapid test

Meat is a basic component in the human diet, one of the main foods consumed worldwide. The nutritional quality of meat is given by proteins with high biological value, lipids, vitamins and minerals.

Average meat consumption worldwide is estimated at 42.9 kg per capita, in developed countries meat consumption is about 76.1 kg meat per capita, twice the amount of meat consumed in developing countries (33.6 kg per capita) (6). It is estimated that 80% of farm animals are treated with veterinary drugs during their life cycle (1, 15). Moreover, antibiotic consumption is expected to increase by 11.5% in the livestock sector by 2030 (17). In fact, antimicrobial substances are frequently used in farm animals for the treatment of various bacterial infections. Failure to comply with the waiting periods recommended by the manufacturer leads to their persistence in meat in the form of drug residues. The presence of residual antimicrobial compounds in meat is a potential danger to the consumer, as these substances can lead to imbalances in the intestinal flora or promote the occurrence of antibiotic resistance in the intestinal microbiome, especially in gram-negative bacteria. Also, residual drugs, especially antibiotic residues, induce allergic reactions and even anaphylactic shock in sensitive people. Along with the immunopathological effects, a number of other harmful effects are recognized, such as carcinogenic, mutagen and teratogen potential. Last but not least, the irrational use and abuse of antimicrobials in food-producing species contributes to the spread of microorganisms with antibiotic resistance in the environment (4, 6, 11, 14, 17). Although the European Union has introduced maximum limit values for residues of various antibiotics in milk and meat (21), this does not guarantee the absence of antimicrobial residues in food of animal origin (20).

Over time, rapid methods of detecting antibiotic residues in meat have been developed, which provide results in a short time, are easy to apply and do not require expensive equipment. They cannot replace the reference methods for the identification and quantification of antimicrobial residues, but could be used to monitor the presence of antibiotics in meat, in the food chain, in particular in self-monitoring programs of food producers, and also in the processing and marketing stages. In this context, the present study aimed to detect antibiotic residues in meat intended for public consumption, using a rapid microbiological test.

Materials and methods

Sampling of meat. The research was carried out on raw meat (sliced meat, minced meat and raw sausages) Samples of red meat (pork, beef) were purchased from various retail stores. Thus, of the 48 samples collected, 28 samples were pork, namely leg, neck, chop, chest, ribs, organs (heart and liver), and sausage; 16 meat samples beef, namely leg, sirloin, rib eye steak, shank cross cut); 2 samples of minced beef-pork mixture and 2 samples of mutton (leg). After collecting the samples, they were individually packed in sterile bags, identified and stored in refrigerated conditions. Then, the samples were transported to the laboratory of Food hygiene and technology.

Detection of antibiotic residues in meat. The analysis of meat samples for the detection of antibiotic residues was performed using the rapid microbiological test - MeRa Test, which use *Geobacillus stearothermophilus* as indicator microorganism, according to the manufacturer's instructions.

Briefly, a quantity of 2 grams was taken from each sample of minced meat, which was placed in a test tube, over which a volume of 6 ml of distilled water was added, the meat / water ratio being 1/3. the pH of the liquid was checked at each sample to ensure an optimal pH of 5.6. The pH of the liquid was checked at each sample to ensure an optimal pH of 5.6. In samples with inadequate pH, it was intervened, for correction, either by adding a solution of 0.1 N NaOH or 0.1 N HCl, to alkalize or to acidify the aqueous meat extract. The correction of the pH is recommended by the manufacturer, being necessary to eliminate the false-positive or false-negative results, as too acidic a pH leads to a yellow turn and implicitly to a false-negative result (invalidation of the test). On the contrary, the alkaline pH delays a possible turning of the color to yellow, in the presence of antibiotics in the sample. After homogenization of the contents, the samples were centrifuged (4000 rotations per minute) for 15 minutes. The obtained supernatant was transferred to test tubes. A disc impregnated with *Geobacillus stearothermophilus* spores was inserted into each test tube, followed by their homogenization. The next step consists in the incubation of the samples, at 64°C for 20 minutes, to allow germination and proliferation of *G. stearothermophilus* cells. One ml was taken from the supernatant obtained for each sample, which was introduced into the test tube with culture medium, then it was closed with a lid. The samples thus prepared were left at room

temperature for 20 minutes. The tubes were then reintroduced to the thermostat, at 64°C for 3.30 hours.

MeRa test allows the qualitative detection of residues of 29 antimicrobial agents, belonging to 9 classes, namely: betalactams, tetracyclines, macrolides, lincosamides, aminoglycosides, sulphamides, sulfanilamides, benzil pirimidine, quinolones, within the limits specified by the EU legislation (21, 23).

Results and discussions

MeRa Test is a qualitative test for detecting antibiotics in meat. The interpretation of the results was made based on the color obtained at the end of the incubation period. Thus, the purple samples were considered positive, indicating the presence of antibiotic residues in meat (grow inhibition of *Geobacillus stearothermophilus* cells). On the other hand, the yellow color was interpreted as a negative result (absence of antibiotic residues). In this case, the change of color, from purple to yellow, means the multiplication of bacterial cells of *Geobacillus stearothermophilus*, marked by the decrease of pH values, due to the fermentation of sugars from the culture medium.

At the end of the incubation period, of 3.30 hours, out of the total of 48 samples tested, 24 (50%) samples had a positive result. Of these, 18 (37.5%) samples were pork, 4 (8.3%), beef, and 2 (4.2%), mutton (Table 1).

Table 1

Detection of antibiotic residues in meat (MeRa test)

No.	Meat samples origin	No. of samples tested (species)/ no. of positive samples	% of the total number of samples tested
1.	Pork	30/18	**37.5**
2.	Beef	16/4	**8.3**
3.	Mutton	2/2	**4.2**

Due to the large number of positive samples, the incubation period was prolonged to 6 hours.

At the end of this period 14 samples had a positive result (29.2%), 6 had a doubtful result (12.5%), having a greenish color, and 28 samples were negative (58.3%) (Fig. 1, Table 2). Reducing the number of positive samples after prolonged incubation may be caused by the presence of small amounts of antimicrobials, below the test detection limit.

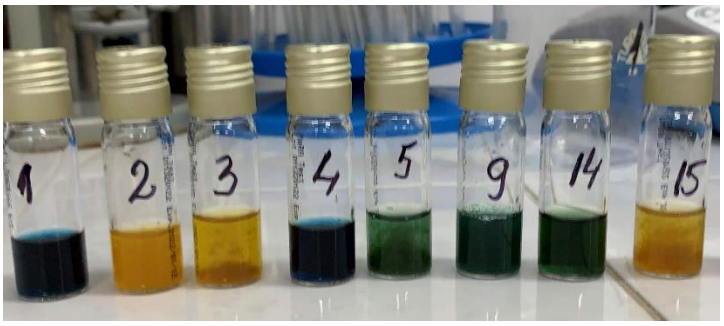

Fig. 1. Negative result - yellow samples (2, 3, 15); positive result purple samples: (1, 4); doubtful result: greenish samples (5, 9, 14)

Heat treatments that are applied to meat, in the process of processing into meat products or cooking, can reduce the risk of ingestion of antimicrobial substances, such as sulfonamides, tetracyclines and fluoroquinolones, but do not guarantee the breakdown of these compounds (20).

Table 2

Antibiotic residues in meat after prolonged incubation (MeRa test)

No.	Meat samples origin	No. of positive samples (%)	No. of doubtful samples (%)
1.	Pork	9 (18.7%)	5 (10.4)
2.	Beef	4 (8.3%)	1 (2.1)
3.	Mutton	1 (2.1%)	0

The large number of positive results is a public health concern. It has been reported that levels of antimicrobial administration correlate strongly with the level of resistance to these agents in commensal (2, 3), or even zoonotic bacteria (13), isolates from meat.

In EU member states the percentage of non-compliant foods, with concentrations of antimicrobial substances exceeding the maximum allowed limit, is extremely low (0.14 %) (22). However, in Romania, the occurrence of bacteria with multiple antibiotic resistance in food of animal origin and in slaughterhouse environment it has been reported in the last decade (5, 7, 12, 16, 18, 19). In the context of an integrated One Health approach, in the last decade, important progresses have been achieved in the monitoring of the biological safety of the red meat in Romania (8, 10), especially in western counties. In addition, another study conducted in the same region underlined that the widespread of zoonotic pathogens in the surrounding livestock environment, such as surface waters (9) can greatly contribute to the occurrence of several disease with negative impact on the quality

of animal productions.

In this study a small number of samples were analyzed. In order to know the real situation, additional investigations are needed, on an increased number of samples. Also, further investigations are needed on the positive samples, to quantify the concentration of antibiotic residues in meat.

Conclusions

The presence of antibiotic residues may pose a public health risk and a warning to the authorities responsible for the control of food of animal origin.

Obtaining negative results, after prolonged of the incubation period, in a large proportion of samples that were initially positive, could be the consequence of the presence of residual quantities of antimicrobials below the detection limit of the microbiological test.

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THE EFFICACY OF TWO ESSENTIAL OILS ON *STREPTOCOCCUS PYOGENES*

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Summary

The emergence of antibiotic resisting *Streptococcus* species and treatment failure has become a major problem worldwide. The pathogen is known to cause a variety of infections throughout the body, ranging from moderate localized infections to even life-threatening systemic infections. Treatment of infections caused by drug resisting bacteria with natural antimicrobial constituents such as natural plant compounds is considered as a safer option nowadays due to their lesser side effects and better prognosis. The aim of the present study is to evaluate the antimicrobial activity of two essential oils, *Pogostemon cablin* (Patchouli), and *Pinus sylvestris* (Pine), on a strain of *Streptococcus pyogenes* using the broth microdilution technique. In order to better understand the activity of the oils, we tested them separately, and as a mixture. The results of this study were determined using the ELISA method and they proved that the essential oils exhibited antimicrobial activity against *Streptococcus pyogenes*, at different concentrations and in both scenarios. The *Pogostemon cablin* essential oil exhibited the highest antibacterial effect against the pathogen, while the activity of *Pinus sylvestris* essential oil was maintained at relatively constant levels. From an economical point of view, the patchouli essential oil is more expensive than the pine oil, thus, by mixing them we obtained a cheaper version of a potent treatment against *Streptococcus pyogenes* antimicrobial resisting strains.

Keywords: *Streptococcus pyogenes*, *Pogostemon cablin*, *Pinus sylvestris*, essential oils, antimicrobial

The *Streptococcus* genus includes more than 70 species, from which *Streptococcus pyogenes* is the most important (15). *S. pyogenes* is one of the major bacterial pathogen that causes a wide variety of clinical manifestations, ranging from moderate localized infections to even life-threatening systemic infections (4, 6). The pathogen is also known as group A *Streptococcus* (GAS), and is characterized as a gram-positive, non-motile, non-spore forming, catalase-negative and beta-haemolythic bacteria (11, 13).

The European *Pinus sylvestris* (Scots pine) is a tertiary relict belonging to the group of Mediterranean pines. It is one of the most widespread and polymorphic conifers in Europe (3, 12). The pine essential oil has antimicrobial actions, which is why it is used as antimicrobial additive for manufacturing medicinal products and cosmetic products (1, 7, 8).

Pogostemon cablin (Patchouli plant) has been widely used worldwide as ingredient in cosmetic products (10), natural food flavoring additive, and more importantly, the patchouli essential oil is used in medicine to strengthen the immune activity and the resistance to bacterial actions (2, 5, 14).

The aim of this research was to observe the antimicrobial activity of the *P. sylvestris* and *P. cablin* essential oils against planktonic *S. pyogenes*. Therefore, the plant extracts have been used both separately and combined (9). This study was inspired from the lack of evidence regarding the antimicrobial action of the two essential oils against the pathogen *Streptococcus pyogenes*, and due to the raising worldwide problem of antibiotic resisting *Streptococcus* species. The results of the study will add value to the medicinal properties of these herbs, and urge these essential oils to be taken forward as innovating agents against drug resistant bacteria. However, more research is required to identify the active compounds of these plants in order to develop new antimicrobials.

Materials and methods

In order to conduct the research, we needed the following equipment: Elisa reader (Bio-Rad PR 1100); Brain Heart Infusion (BHI) broth (Oxoid, CM1135); BHI agar; *S. pyogenes* Rosenbach (ATCC 19615); Thermostat; 96 sterile well plate and a Calibra Digital 852 multichannel pipette.

The essential oils of *P. cablin* (PC) and *P. sylvestris* (PS) were tested in vitro for antimicrobial activity against *S. pyogenes* Rosenbach (ATCC 19615), using the broth microdilution method (ISO 20776-1:2019). Therefore, a 10^{-3} dilution of the fresh *S. pyogenes* culture was used to perform the assay, an inoculum equivalent to a 0.5 McFarland standard. The ATCC strain was revived by overnight growth in Brain Heart Infusion (BHI) broth (Oxoid, CM1135), at 37°C and, subsequently, passed on BHI Agar, for 24 hours at 37°C. The bacterial strains were then diluted at an optical density (OD) of 0.5 McFarland standard (1.5×10^8 UFC/mL) using BHI broth.

Afterwards, 100 µL from the diluted strain were placed in each well corresponding to the 96 microdilution well plate, using a Calibra digital 852 multichannel pipette. The essential oils were tested directly by placing 2 µL, 4 µL, 8 µL, 16 µL and 32 µL in each well of the plate, furthermore, triplicate tests were performed for all samples. The suspension of strain and BHI was used as a positive control. The plates were then covered and left overnight at 37°C.

The optical density (OD) was measured at 540 nm using the Bio-Rad PR 1100 apparatus, which is an ELISA reader. Several measurements were done, as follows: the initial reading; after one hour; after six hours; after 12 hours; after 24 hours.

Results and discussions

The essential data was gathered taking into consideration a precise timetable, as follows: the initial reading (right after the putting the oils in the mixture of broth and bacteria), after one hour, six hours, 12 hours, and 24 hours respectively.

By doing this, we covered all the growth phases of *S. pyogenes* and observed the antibacterial action of the oils in each of them (Table 1).

Table 1

The results expressed in growth factor and efficacy

Samples	Moment of reading the results									
	Initial		1 hour		6 hours		12 hours		24 hours	
	Growth factor (%)	Efficacy	Growth factor (%)	Efficacy	Growth factor (%)	Efficacy	Growth factor (%)	Efficacy	Growth factor (%)	Efficacy
P 2µL	107.17	7.17	100.36	0.36	59.89	-40.11	94.85	-5.15	92.99	-7.01
P 4µL	104.78	4.78	93.86	-6.14	40.17	-59.83	82.35	-17.65	98.16	-1.84
P 8µL	108.76	8.76	101.44	1.44	38.38	-61.62	95.67	-4.33	96.62	-3.38
P 16µL	114.34	14.34	107.58	7.58	23.24	-76.76	86.41	-13.59	94.48	-5.52
P 32µL	133.07	33.07	121.30	21.30	25.70	-74.30	17.79	-82.21	21.32	-78.68
L 2µL	158.96	58.96	145.85	45.85	19.19	-80.81	12.96	-87.04	14.26	-85.74
L 4µL	193.63	93.63	207.58	107.58	19.85	-80.15	12.91	-87.09	14.31	-85.69
L 8µL	214.74	114.74	247.65	147.65	21.45	-78.55	14.13	-85.87	16.35	-83.65
L 16µL	253.78	153.78	219.13	119.13	20.19	-79.81	13.81	-86.19	15.56	-84.44
L 32µL	370.92	270.92	289.17	189.17	38.51	-61.49	15.53	-84.47	19.88	-80.12
P+L 4µL	120.32	20.32	105.78	5.78	18.73	-81.27	12.78	-87.22	14.81	-85.19
P+L 8µL	124.70	24.70	114.08	14.08	21.25	-78.75	14.63	-85.37	16.10	-83.90
P+L 16µL	127.09	27.09	110.83	10.83	20.45	-79.55	13.72	-86.28	14.71	-85.29
P+L 32µL	183.67	83.67	128.52	28.52	21.51	-78.49	14.54	-85.46	15.76	-84.24
S. pyogenes	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
Average OD	0.08		0.09		0.50		0.74		0.67	

Growth factor – the percentage of strain growth compared to the positive control; Efficacy – The – and + strain inhibition percentage; OD – Optical Density; P – *P. sylvestris*; L – *P. Cablin*

Analyzing the average optical density, we can summarize that *S. pyogenes* is within the lag phase, starting from when the inoculum is added to the culture media, until after one hour of incubation, as observed during the first two readings. After six hours of incubation the results indicate that the pathogen's cells are in the log phase, also known as logarithmic growth. After twelve hours, at the fourth

reading, *S. pyogenes* reached the stationary growth phase, since no substantial difference was noticed in their growth rate, in comparison to the previous reading. The decrease of optical density observed at the fifth reading (after 24 hours of incubation) shows that the pathogen is in the decrease and cell death phase.

After collecting all of the data, we had a larger overview of our essential oils' antimicrobial activity.

In the case of *Pinus sylvestris aetheroleum*, the antimicrobial effect was observed at concentration of 32 µL, but the other concentrations had almost no effect, as represented in the following graphic (Fig. 1).

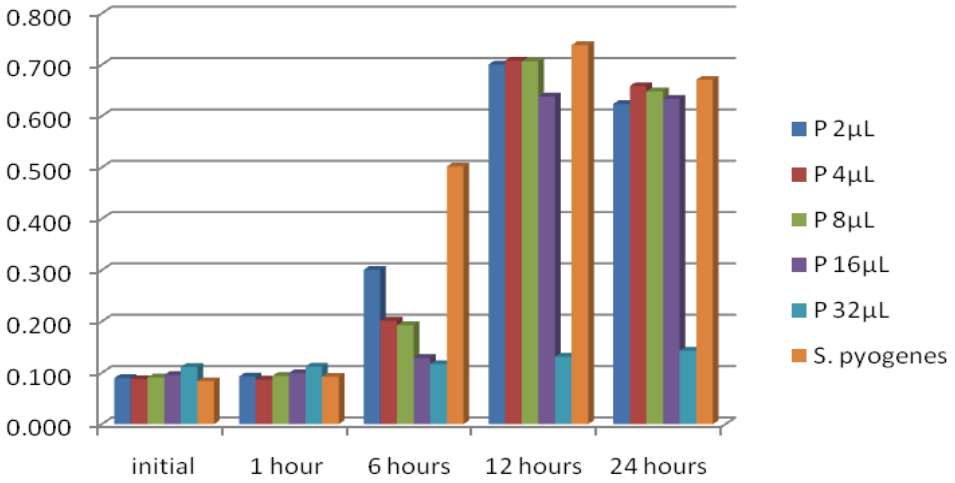

Fig. 1. Antimicrobial effect of the *Pinus sylvestris aetheroleum* (P) against *S. pyogenes* in comparison with the positive control

Regarding the antistreptococcal action of *Pogostemon cablin aetheroleum*, the data shows that it performs greatly starting from concentrations as low as 2 µL (lowest dose). Due to the darker color of the oil, the initial results are slightly influenced, but the antimicrobial effect can be clearly noticed during the third reading (at six hours after inoculation), which corresponds to the log phase (Fig. 2).

In the following, we will present the *P. sylvestris* and *P. cablin aetheroleum* mixture's effect against the pathogen strain. As presented in the following graphic, the two essential oils potentiated each other, therefore enhancing the antistreptococcal effect (Fig. 3). The synergy between them is suggesting that the patchouli essential oil is boosting the effects of chemical components in the pine oil.

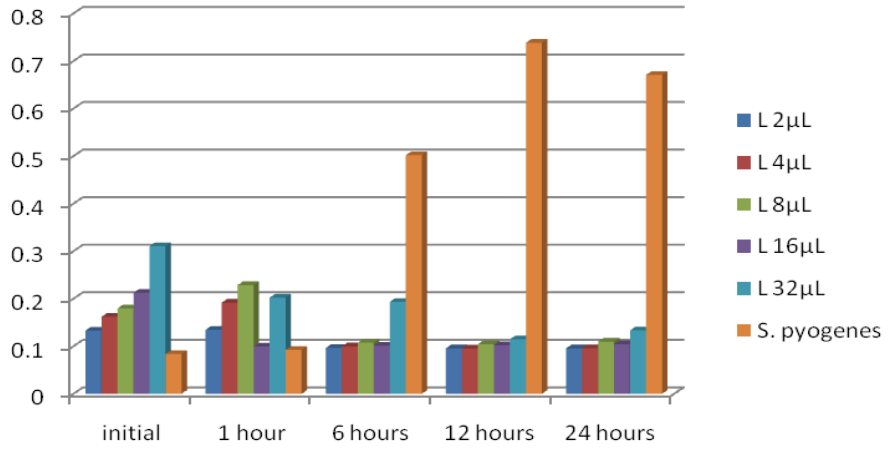

Fig. 2. Antimicrobial effect of the *Pogostemon cablin aetheroleum* (L) against *S. pyogenes* in comparison with the positive control

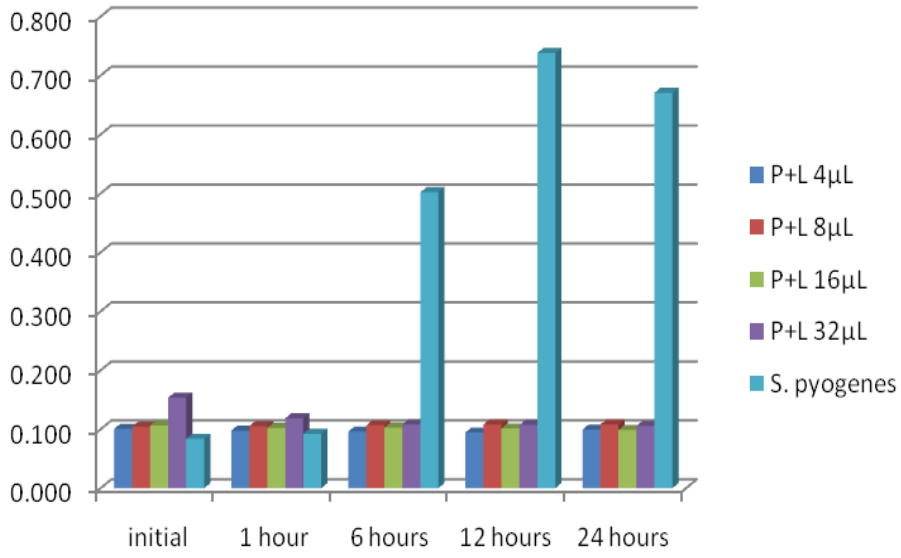

Fig. 3. Antimicrobial effect of the *Pinus sylvestris* (P) and *Pogostemon cablin aetheroleum* (L) mixture against *S. pyogenes*

Besides the discovery of a potentially new treatment for infections caused by antimicrobial resistant *Streptococcus pyogenes* strains we also obtained a cheaper version of it, taking into consideration the fact that the patchouli essential oil

is more expensive than the pine oil, thus, by mixing them we reduce the costs to make it.

We are living times where the occurrence of antimicrobial resistance amongst bacteria is progressive. The exaggerated daily use of antibiotics in homes, hospitals, and even agriculture is unquestionably responsible for this crisis. In order to cure diseases caused by multidrug resistant bacteria, the use of natural essential oils quickly expanded.

Conclusions

This study revealed that *Pinus sylvestris aetheroleum* and *Pogostemon cablin aetheroleum* manifest inhibitory action against *Streptococcus pyogenes*, which is a pattern for the Gram-positive bacteria.

ELISA method approves that, used alone the patchouli essential oil had the most promising antibacterial results against *S. pyogenes*, compared to the pine essential oil which showed approximately constant effect despite of the used concentration. On the other hand, the mixed oils acted in synergy, therefore enhancing the antistreptococcal activity.

The analyzed mixture of essential oils could be subject to further studies regarding its utilization as antimicrobial drug in infections caused by *S. pyogenes*.

Our research let us discover besides a new potent treatment for *S. pyogenes* resistant strains, also a cheaper version, economically speaking, by mixing the two oils, as obtaining patchouli essential oil is more expensive than pine oil.

Acknowledgements

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MASTECTOMY IN CATS AND DOGS – A REVIEW

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Summary

Tumors arising from mammary tissue are one of the most common mammary gland disorders observed in small animal practice. This pathology interferes with the normal functioning of this exocrine gland, with the offspring health and with the general condition of the patient, thus a correct management will improve the overall survival. Surgical intervention is the most widely accepted option of treatment for mammary gland tumors, besides chemotherapy or radiation therapy, because the complete surgical removal of localized cancer can cure more patients than any other therapy. The surgical technique selection for the tumor removal varies according to mass location, size, stage, consistency, patient status, the surgeon preference and sometimes the species characteristics, such that, the same tumor type can present a different surgical approach. This review aims to summarize the main surgical techniques in mastectomies and their selection criteria, because usually the surgeon has to select between lumpectomy, simple mastectomy, regional mastectomy, unilateral mastectomy or bilateral mastectomy.

Keywords: mastectomy, tumors, small animal, surgical technique

Mammary neoplasia is an important disease entity in veterinary medicine, frequently diagnosed in female dogs and cats (12). Tumors of the mammary gland are the most common tumor in the female dog, but less frequent in cats, even they account for nearly one third of all feline tumors (7). The mammary neoplasm incidence varies according to the country involved, due to the different timing of the neutering. For instance, in North America the incidence is lower than in many others regions, because the ovariohysterectomy is done earlier (4).

The etiology of this pathology is unknown. In both dogs and cats however, three main factors play important roles in mammary neoplasia: age, hormonal exposure and breed (20). Hormonal exposure, primarily during mammary gland development, is the major risk factor. Estrogens and progestins stimulate mammary tissue growth and may influence tumorigenesis through direct growth factor activity (4). A large percentage however might be prevented by performing the ovariohysterectomy in the first year of life, because many are hormone-dependent. The neutered dogs and cats have approximately seven times lower risk of developing mammary tumors compared to intact animals (7).

Progestin-treated dogs and cats are more likely to develop tumors, having an overall relative risk of 2.3, respective 3.4 compared to those not receiving such treatment (20).

In dogs, osteosarcoma is the most common mesenchymal tumor of the mammary gland, and usually the anamnesis indicates a rapid growth of a mammary mass that was present for some time (20). The inflammatory carcinomas should not be excised, because the prognosis is too poor (7).

According to the California Animal Neoplasia Registry, mammary gland neoplasia, after skin tumors and lymphoma represent the third most common tumor type in cats. As in the dog, mammary tumors are generally seen in middle-aged to older cats, usually 10 to 12 years. In particular, Siamese cats are diagnosed with this disease at a significant younger age, the peak risk being at 9 years (20).

Materials and methods

Clinical assessment

Tumors can be easily detected on physical examination. They usually have the aspect of a firm, singular or multiple, intact or ulcerated mass on the mammary gland (Fig. 1).

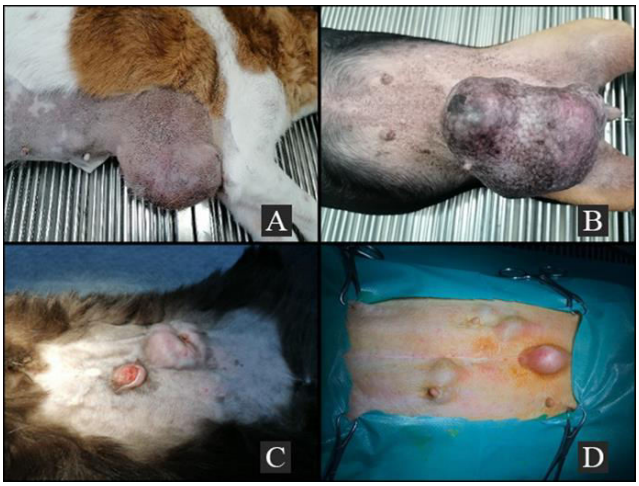

Fig. 1. Clinical aspect of the mammary gland tumors: A. Thoracal mammary neoplasia in bitch. B. Inguinal mammary tumor in bitch. C. Multiple ulcerated mammary tumors in cat. D. Multiple mammary tumors in bitch

Considering the risk of metastasis that occasionally comes along with mammary tumors, it's strongly recommended to assess and stage the disease, prior initiating the therapy. According to Vail et al. (20), a basic staging includes serum biochemistry, complete blood count, three-view thoracic radiographs and fine-needle aspiration of regional lymph nodes, even if they are normal on palpation. CT imaging of the thorax provides a more accurate detection of pulmonary nodules than does

thoracic radiography, but has some disadvantages such as increased expense, decreased availability and need for general anesthesia.

The prognosis is usually associated with three factors: tumor size, lymph node involvement and distant metastasis (20). Tumor size has been found to influence the prognostic, such that this delineates the first three stages. In dogs, lymph node metastases determine stage four regardless of tumor size, and distant metastases indicate stage five (4). The description of these prognostic factors is beyond the scope of this review, thus a brief explanation of World Health Organization staging scheme is presented in Table 1.

Table 1

Staging Systems for canine and feline mammary tumors (4)

Modified World Health Organization Tumor, Node, Metastasis (TNM) staging Scheme						
Canine mammary tumor staging				Feline mammary tumor staging		
Tumor size	**Lymphnode metastasis**	**Distant metastasis**	**Stage**	**Tumor size**	**Lymphnode metastasis**	**Distant metastasis**
T1 (T<3 cm)	N0 (none)	M0 (none)	**1**	T1 (T<2 cm)	N0 (none)	M0 (none)
T2 (T 3–5 cm)	N0 (none)	M0 (none)	**2**	T2 (T 2–3 cm)	N0 (none)	M0 (none)
T3 (T>5 cm)	N0 (none)	M0 (none)	**3**	T3 (T>3 cm) Any T (T1 or T2)	Any N N1 (positive)	M0 (none) M0 (none)
Any T	N1 (positive)	M0 (none)	**4**	Any T	Any N	M1 (positive)
Any T	Any N	M1 (positive)	**5**	x	x	x

Surgical anatomy

Dogs usually have five pairs of mammary glands (two thoracic, two abdominal and one inguinal), arranged in two rows, while cats have four pairs (two thoracic and two abdominal) (6). Glands are supplied by cutaneous branches of the intercostals, branches of the lateral and internal thoracic vessel, and by the cranial and caudal epigastric arteries. The venous drainage of the mammary glands parallels the arteries (18). Regarding the lymphatic drainage, the three cranial glands drain to the axillary lymph nodes, and the inguinal lymph node drains the two caudal glands (7).

Therapy

Surgery is an integral part of therapy for mammary neoplasm, usually the treatment of choice, with the exception of those with widespread metastases or the inflammatory carcinoma (7).

The surgical techniques that can be performed for mammary tumor removal are lumpectomy, simple mastectomy, regional mastectomy, unilateral mastectomy and bilateral mastectomy (Table 2). Surgical technique selection and the extent of

surgical excision are individualized for each patient, considering the size of the primary tumor, its location, adherence, co-existing diseases and the probability to achieve local tumor control (2, 14). The surgical margin assessment is crucial in this sort of intervention, and so, an additional surgery should be pursued if the removal is incomplete (20).

If the patient has several tumors in both chains, a combination of different techniques may be chosen (Fig. 3). Also, if it's not possible to perform a complete excision in a single surgery, a second procedure should be delayed, until the skin is relaxed and healed (7).

The animal is placed in dorsal recumbency, and the caudal thorax, entire ventral abdomen or/and the inguinal areas are clipped and prepared for aseptic surgery (7).

The animal is placed in dorsal recumbency, and the caudal thorax, entire ventral abdomen or/and the inguinal areas are clipped and prepared for aseptic surgery (7).

Table 2

Surgical techniques for mammary neoplasms removal (7, 18, 19)

Type of surgery	Lumpectomy	Simple mastectomy	Regional mastectomy	Unilateral mastectomy	Bilateral mastectomy
Description	The excision of a small mass (≤5mm), with a surrounding margin of normal appearing tissue (1 cm)	Resection of the entire gland containing the tumor.	Removal of the involved and adjacent glands.	Resection of the tumor with all the ipsilateral glands	Resection of all mammary tissue
Indications	Usually when the mass is located at the gland periphery	Usually when the neoplasm involves the central area of the gland, or most of it.	When: - multiple tumors occur in adjacent glands - tumor occurs between two glands	When: - multiple tumors occur throughout the chain - *a single mass is present, because the local recurrence is frequent in cats	When multiple masses occur in both chains
Contraindications	Multiple lesions or signs of malignancy	Multiple lesions or signs of malignancy			High tension on the wound, therefore it's recommended to be staged.

Lumpectomy refers to small nodule removal. This procedure is performed by incising the skin over the nodule which is grasped and bluntly isolated using mosquito hemostats or scissors. The disadvantage of this technique is that it often leads to tumor recurrence, therefore should be avoided (2, 14).

Single mastectomy (Fig. 2) is the most commonly used technique, indicated for large tumors with a central location within the gland. A circular skin incision, with 2 cm margins of unchanged, healthy tissue is made around the gland, following that if the abdominal muscle fascia or underlying muscle is involved, all should be excised en block. If they are not involved, the glands are stripped from the tissue, only by gentle traction. However, first two cranial glands removal may require sharp and blunt dissection of the pectoral musculature, whereas for glands three to five, the dissection is easier. The medial boundary of the incision should be the ventral midline. Sometimes it's less traumatic and simpler to make a regional mastectomy, removing both adjacent glands, rather than trying to divide merging mammary parenchyma between them (2, 7, 13).

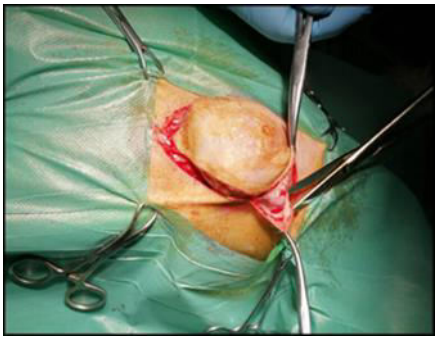

Fig. 2. Skin incision in simple mastectomy

In regional mastectomy (Fig. 4), an elliptical incision around the mammary glands is made then continued through subcutaneous tissue with blunt dissection using surgical scissors. Traction should be used on the elevated skin segment, to facilitate the dissection. This is a simple procedure if it involves the abdominal and inguinal glands that are loosely attached by adipose and connective tissue, but becomes much more difficult on the thoracic glands which adhere to the underlying pectoral muscles. Also, the skin apposition is more difficult in the thoracic region, because of the low mobility of the skin (7). If possible, incising the mammary tissue should be avoided.

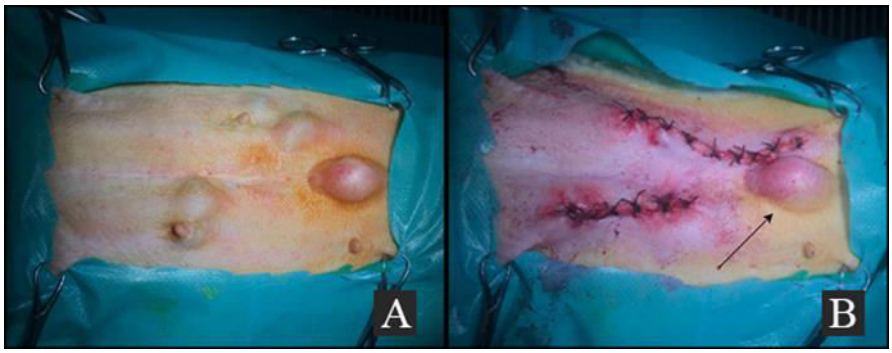

Fig. 3. Bilateral simple mastectomy. A. Clinical aspect of the tumors before surgery. B. Postoperative aspect; The black arrow indicates an umbilical hernia

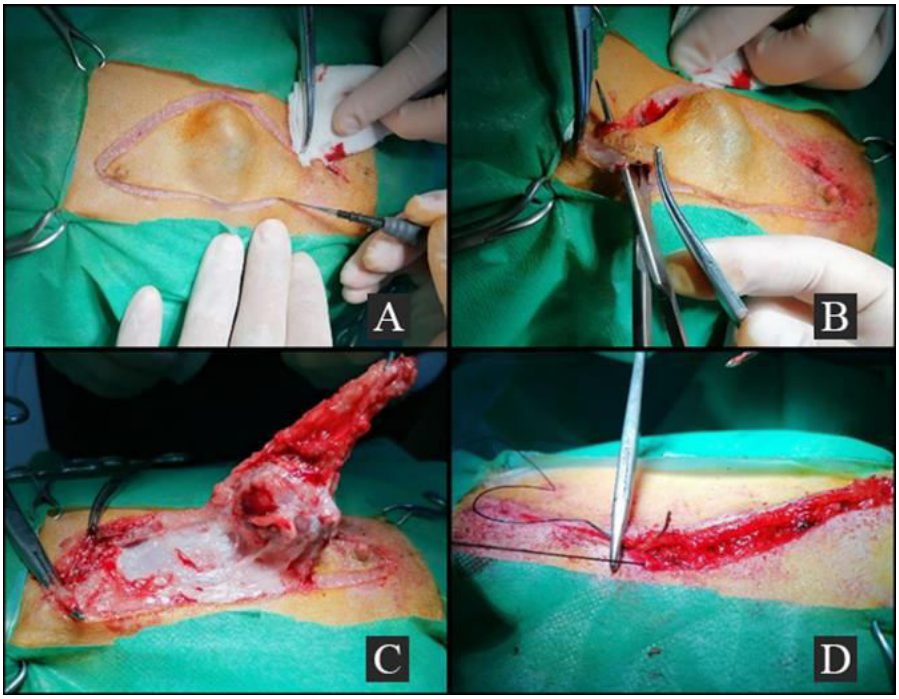

Fig. 4. Regional mastectomy. A. Elliptical skin incision. B. Isolation of the vascular cord and transfixic ligature preparation. C. Using traction to facilitate the dissection. D. Subcuticular suture

Care should be taken to the major supplying vessels (Table 3). After separating the subcutaneous tissue from the fascia, these vessels will be isolated and ligated, before the entire mass is removed. The surgical wound closure is considered to be one of the most challenging part of the procedure because of the wide spaces that are left. Due to the large mastectomy wounds and the considerable tension that accompanies it, the skin edges are advanced to the center of the defect using walking sutures, and then subcuticular sutures (16). The skin is apposed with simple interrupted or cruciate pattern, using a nonabsorbable suture, usually monofilament nylon (7).

Table 3

The major supplying vessels of the mammary glands (7, 19)

	Mammary glands		
	1 & 2	**2 & 3**	**4 & 5**
Major supplying vessels	Branches of the intercostal, internal and lateral thoracic vessels	Cranial superficial epigastric vessels	Caudal superficial epigastric vessels *External pudendal vessel – that connects the gland 5 caudally
Surgical approach	Vessel ligature where it penetrates the rectus abdominis	Vessel ligature where it penetrates the rectus abdominis, between the caudal and cranial abdominal mammary glands.	Vessel ligature, adjacent to the inguinal ring.

When tumors invade the subcutaneous tissue, muscular fascia or portions of the body wall will be included in en bloc resections (20).

A more extensive surgical resection, such as unilateral or bilateral chain mastectomy, may need to be pursued in animals with multiple mammary tumors. This implies removal of glands 1 to 5 of a chain, as a unit. When multiple neoplasms are located in both chains, a bilateral mastectomy may be performed (3, 14). Recommendations for surgical interventions of this magnitude are more indicated in young, intact females with multiple tumors, as they have a higher risk of developing additional neoplasms (20).

Some authors recommend a routine removal of the superficial inguinal lymph nodes, in regional and unilateral mastectomy that includes the inguinal mammary gland, since they are embedded in the fat attached to this gland. Even if the resection of axillary lymph nodes is similarly recommended when cranial mammary glands are affected, the removal is seldom performed due to the brachial plexus and the difficult access (18).

If ovariohysterectomy has to be performed simultaneously, tumor removal prior to abdominal opening has to be avoided, in order to prevent direct spread of tumor cells; thus, tumor excision should follow the abdominal closure (20).

The potential complications after mastectomy are inflammation, pain, seroma formation, hemorrhage, infection, ischemic necrosis, dehiscence, self-trauma, rear limb edema, or tumor recurrence (1, 7).

Postsurgical care

Analgesia is a critical element of the postoperative management in animals undergoing mastectomy. A good analgesic protocol will improve outcome and recovery time (2). Also, supportive care, Elizabethan collar, and a periodically inspection of the skin wound for inflammation, swelling, or dehiscence, is needed. Some authors recommend suction drainage, although, in a study on sixty dogs, no significant differences were found among the evaluated groups, regarding the presence or absence of the drain (13, 14).

Systemic therapy

Early diagnosis and aggressive surgery usually lead to long-term survival of animals with early stage mammary neoplasia, but when the diagnosis is delayed, primary tumors are large or lymph node metastasis are present, systemic treatment is also needed in addition to surgical treatment (20).

Despite the fact that few clinical studies investigated systemic therapy for mammary gland tumors, systemic treatment is routinely recommended and administered in dogs with high-risk tumor (20).

Hormonal dependence of the tumors underlies the use of hormone therapy in dog mammary gland neoplasia. This might be achieved by medical means (specific ER modulators – SERMs- and aromatase inhibitors or luteinizing hormone-releasing hormone agonists, last two suppressing estrogen secretion) or surgical procedure (ovariectomy). The results are however conflicting. For instance, dogs with large primary tumors, lymph node involvement and undifferentiated histology are less probably to have HR-positive tumors, and so they are less likely to be advantaged by the hormonal ablation (20).

Considering the low HR expression in feline mammary carcinoma, the hormonal treatment has a low efficiency in this disease (20). In dogs, nonsteroidal anti-inflammatory drugs seem to be useful in inflammatory carcinomas, according to Marconato L. et al. (10) and Souza et al. (17).

Efficacy of chemotherapy in cats with mammary cancer is controversial; some have shown a positive response in almost 50% of the cases, while others obtain a shorter median time of survival after chemotherapy, than in cats treated with surgery alone. Doxorubicin, cyclophosphamide, or combination was used (20).

Chemotherapy is regularly administered in dogs with mammary tumors at risk of recurrence or metastasis. Karayannopoulou et al. (8) found in 2001 that dogs, with stage III or IV mammary tumors, show a significant survival benefit after receiving a combination of 5-fluorouracil and cyclophosphamide adjuvant to surgery, in contrast to patients treated with surgery alone.

Results and discussions

Exposure to ovarian hormones or exogenous progestins, even estrogens, is strongly implicated in mammary tumorigenesis in dogs and cats. There are many controversial opinions regarding the risk percentage of developing mammary tumors, depending on the exact time of ovariohysterectomy (5). However, in dogs, there is a general agreement that the major benefit in the prevention of the mammary neoplasia is achieved if spaying is done before the onset of the first heat cycle. This suggests that the irreversible ovarian hormone effects on the mammary glands, in terms of cancer risk, occur early in life during the mammary gland development (18, 20).

A study from 2005 concluded that surgery alone can cure benign mammary tumors and almost 50% of malignant tumors, because the other 50% of the patients already had micrometastasis when the surgical intervention was made. So, despite surgery, the disease will progress and lead to the patient's death (18).

Most studies indicate that for dogs with a single neoplasm, the outcome is identical for mass removal and radical mastectomy, as long as the resection is complete. When it comes to multiple mammary tumors, a regional, unilateral or bilateral mastectomy is recommended (4).

In a study from 2008, 58% of dogs developed a second tumor, whose occurrence could have been prevented if an unilateral mastectomy have been performed. Therefore, a more radical surgery is advised. At the same time, if all the dogs received unilateral mastectomies, a large amount, 42%, would have had an unessentially aggressive surgery (4). In contrast to these results, a trial of 144 dogs, couldn't show differences regarding the recurrence rate and survival time between simple and radical mastectomy (9).

For mammary carcinoma treatment in cats, some authors recommend a unilateral or staged bilateral chain mastectomy. Miyazaki et al. (11) in an article from 2018 claims that bilateral mastectomy is the most recommended surgical method for feline mammary neoplasia treatment, considering that it significantly diminishes the risk of recurrence, and increases the time of survival. In those with excessively loose mammary tissue it is possible to perform a bilateral chain mastectomy during a single surgical session, with a tolerable postsurgical tension, but the recovery is more difficult and painful (20).

Usually staged bilateral mastectomy in dogs, is performed by taking the unilateral mastectomies on either side, 4 to 6 weeks apart. For single-stage closure in bilateral mastectomies, Miyazaki et al. (11) highlights the usefulness of the skin stretchers. In his case study, a wound closure, without tension or major complication of the patients, was achieved after using skin stretchers in bilateral mastectomy.

The result of the intervention is not necessarily influenced by the technique, unless the excision was not completely made, says Fossum (7). However, recurrence will be lower in animals which have had a unilateral mastectomy, compared to those who have had a lumpectomy (7).

Conclusions

Surgical intervention remains the gold-standard therapy for most types of mammary tumors, but it will be palliative in patients with distant metastases. The right surgical technique is the one that allows a complete tumor removal, with clean margins by using the most simple and atraumatic technique.

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SENSITIVITY TO ANTIFUNGAL SUBSTANCES OF SOME *MICROSPORUM CANIS* STRAINS

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Summary

This study presents the results obtained in testing the sensitivity to certain antifungal substances of some strains of *Microsporum canis* isolated from dogs and cats as well as from human patients. It was found that of the five antifungal substances tested by the diffusimetric method, in terms of efficacy on isolated *M. canis* strains (n = 16), Clotrimazole showed special activity. Compared to all strains tested (16 strains) this substance showed remarkable efficacy, with the diameter of the induced inhibition zones ranging from 22 mm to 40 mm, with an average of 31.12 ± 5.38 mm. With lower efficacy, causing areas of inhibition smaller than Clotrimazole, from intermediate to sensitive, Miconazole and Nystatin acted. There were strains sensitive to Miconazole, but intermediate sensitive to Nistatin, suggesting the need to test the sensitivity to the antifungal substance before starting a treatment.

Keywords: dermatophytosis, *Microsporum canis*, antifungal substances, sensitivity test

Dermatomycoses are of all fungal infections the most common forms of infection in humans, affecting more than 20% - 25% of the world's population (6). It is estimated that in the human population, 30% -70% of adults are asymptomatic carriers of these pathogens (13). From studies conducted so far, it is estimated that zoophilic species are responsible for about 30% of human dermatophytosis and usually cause acute inflammatory conditions. Anthropophilic species account for approximately 70% of infections in these hosts, causing a chronic infection with slow progression, suggesting that the fungus has adapted to the human host (14).

Compared with the importance given to fungal infections in humans, in animals fungal diseases are relatively neglected, even if they are a source of up to 80% of human skin problems in rural areas and 20% of infections in urban areas (16).

Another aspect that must be reported in fungal infections, both in humans and animals is the manifestation of resistance to certain antifungals used in treatment. The most important element that can induce the appearance of antifungal resistance seems to be the improper prescription of systemic antifungal agents and their indiscriminate use (8).

There is no clear evidence of the dosing strategy to be used during treatment and prophylaxis to avoid the best resistance, especially for animals (17).

There are medical studies that have suggested measures to prevent and suppress the occurrence of antifungal resistance, which specify the prudent use of

antifungals and their appropriate dosage, treatment with an antifungal appropriate to the identified etiological agent, after establishing its sensitivity to antifungal substances (5).

In veterinary medicine for the treatment of dermatophytosis in pets there are a limited number of drugs, specially conditioned for dogs and cats. For this are used medical antifungal ointments recommended for the treatment of human dermathosis. Also, for pets systemic treatments of this type of disease, commercially veterinary products are limited, and as result, drugs used for human dermatophytosis treatment are used, but these are difficult to dose.

In addition, it should be noted that due to the transmission of the disease, the owners of animals with dermatophytosis could be also infected. They ask for the help and advice of a veterinarian, for themselves or their family members. As a result it is found that the prescription of topical or systemic antifungal drugs is done without a antifungal susceptibility test, which can lead in time to the development of the resistance on antifungal drugs.

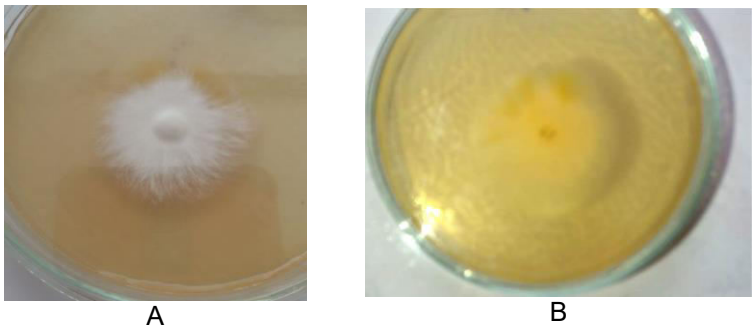
In view of these aspects, the aim of this study was to test the sensitivity of strains of *Microsporum canis*, isolated from various cases encountered in veterinary practices, in order to assess the efficacy of products that are frequently recommended for this infection treatment and to assess the antifungal resistance of this some strains of this dermatophyte specie.

Materials and methods

A total of 16 samples composed of fur, nail and skin scraping specimens were collected from 4 (25%) dogs and 10 (62.5%) cats and 2 (12.5%) humans (pets owner) with clinical suspicion of dermatophytosis, in one veterinary clinic, and analyzed to the Mycology Research Laboratory of the Faculty of Veterinary Medicine from Timisoara.

The skin samples were collected by scraping with a sterile scalpel blade, and the hairs were taken from the center and from the edges of the lesions with a sterile forceps. The samples were deposited in sterile containers for laboratory processing. Their inoculation was performed after their fragmentation with a sterile scalpel blade and immersion in a 70° alcohol solution for 30 seconds. Sabouraud medium with the addition of chloramphenicol, previously poured into sterile Petri dishes, was used for sowing. Incubation of the plates was performed at 27 - 28°C for 7-10 days. The identification of the species of *Microsporum canis* was made on the basis of cultural and microscopic characteristics (11).

Microsporum canis produces colonies that become visible 4-5 days after inoculation, but then grow rapidly. The colonies have a fluffy to woolly appearance, being white-yellow on the obverse and yellow or yellow-orange on the reverse (Fig. 1).



A B

Fig. 1. Microsporum canis - macroscopic characters: obverse (A) and reverse (B)

Direct microscopic examination (wet mount) was performed from pure culture, using lactophenol blue solution. Optika microscope with video camera was used for examination. Initially, the 10 x objective was used for the overall examination of the blade, and then for the magnification of the image and the study of the characteristic elements (macroconidia) the 40 x was used. In the microscopic preparations, the septate mycelial filaments and the typical, fusiform, long, sharp, pointed macroconidia were observed at both ends or with a slightly curved extremity (12). The wall is thick, smooth on some stems or strongly ornamented, even echinous on others. The interior of the macroconidia is puriseptate with 7-14 cells (Fig. 2).

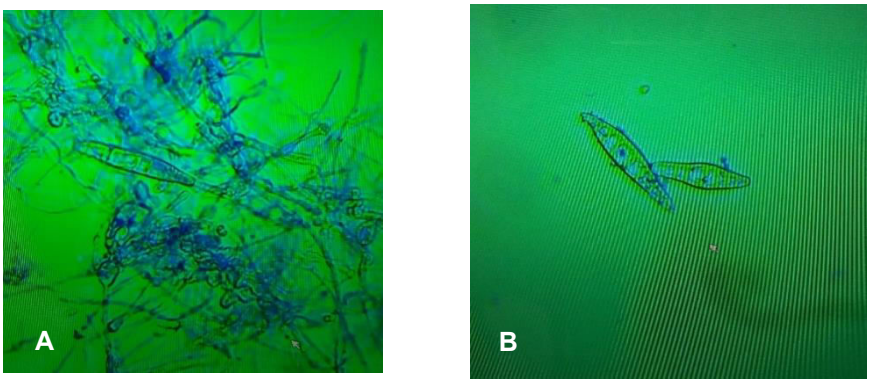


Fig. 2. *Microsporum canis* – microscopic aspect: A - Hyphae and macroconidia *M. canis*, - 10x objective, B - Macroconidia of *M. canis* - 10x objective

Microsporum canis strains antifungal sensitivity testing was performed by diffusimetric method. In this sense, antifungal substances frequently used in this type of dermatophytosis therapy, both in animals and in humans, in topical applications, baths, but also in systemic treatment were chosen. The antifungal substances choosed for this study are: Ketoconazole, Clotrimazole, Miconazole, Amphotericin B and Nystatin (HI Media Laboratories).

Of these, three are substances included in the Azole group (Miconazole, Ketoconazole and Clotrimazole), which act on the synthesis pathway of ergosterol by blocking the activity of the enzyme 14 dimethylase, causing changes in the structure and permeability of the cytoplasmic membrane, and the other two from the Poliene group, which acts by binding to the cell membrane, causing changes in the structure and permeability of the fungal membrane by selective binding to ergosterol (Amphotericin B, Nistatin) (10).

The diffusion method is performed on solid media and is based on the ability of the substances present in the disc to diffuse into the culture medium, from the place where they are deposited. The test is also called a fungigram (18).

In order to perform the test, fresh cultures were prepared from the 16 strains of *Microsporum canis* isolated and preserved in the discipline. For this, inoculations from each strain were made on the Sabouraud medium, with the addition of chloramphenicol, poured inclined (sloping) into test tubes, which were incubated for 7 days at 28°C. A spore suspension was obtained by adding the 10 ml of sterile nutrient broth over the fresh cultures and with the help of the microbiological loop the culture was scraped. The spore suspension thus obtained (at a concentration of 10^6) was used to inoculate on solid medium (Sabouraud with the addition of chloramphenicol). From each test tube, which contained the spore suspension, 1 ml was taken and inoculated by flooding on the surface of the solid medium in the plate. The uniform dispersion of the inoculum on the surface of the medium was then ensured by performing rotational movements with the plate slightly inclined. The plates were then left to stand for 15 minutes to ensure good adhesion of the spores to the surface of the medium, and then sterilely removed, with a pipette, the excess liquid from the surface of the medium. At the end, on the surface of the culture medium, from each plate, the discs with fungicide substance were deposited, respecting the distance of 30 mm between them and 15 mm from the edge of the Petri dish.

After 10 - 15 minutes (pre-diffusion time), the Petri dishes were incubated at 28°C. A first reading of inhibition zone was made after 3-4 days, and the final interpretation after 7 days.

For the interpretation was taken into account the diameter of the inhibition zone, measured in mm with the ruler, in two - three directions, including the disk and the criteria specified by the discs manufacturer (HI Media Laboratories) that present the corresponding values in qualitative attributes: resistant strains, intermediate resistance or sensitive strains (Table 1).

Table 1

Criteria for assessing the action of tested antifungal substances (mm)

Interpretation	Sensitive	Sensitive intermediary	Resistant
Diameter of inhibition zone (mm)	≥ 20	10 -19,9	5-9

Results and discussions

The results regarding the testing of the sensitivity to certain antifungals of some strains of *Microsporum canis* isolated from dogs, cats and human patients collected in a veterinary clinic are presented in Table 2.

Table 2

Efficacy of antifungal substances on some strains of *Microsporum canis*

No. and origin of strain	Diameter of inhibition zone (mm)				
	Miconazole	Ketoconazole	Clotrimazole	Amphotericin B	Nystatin
1 (cat)	21	5	28	5	10
2 (cat)	22	5	31	8	14
3 (dog)	18	5	42	5	12
4 (cat)	17	5	22	6	15
5 (cat)	21	5	27	5	14
6 (human)	22	8	40	5	12
7 (cat)	16	7	31	5	20
8 (dog)	17	11	33	5	19
9 (cat)	18	8	28	7	20
10 (cat)	19	6	28	6	17
11 (cat)	17	7	29	5	20
12 (dog)	21	5	32	5	11
13 (human)	20	10	40	5	14
14 (dog)	18	6	31	7	14
15 (cat)	19	5	29	5	18
16 (cat)	20	5	27	5	13
X ± sdx	19.12±1.89	6.5±1.88	31.12±5.38	5.56±0.96	15.18±3.37

Overall, it was found that of all the five antifungal substances tested by the diffusimetric method, in terms of efficacy on isolated *M. canis* strains (n = 16), Clotrimazole showed outstanding activity. Compared to all strains tested (16 strains) this substance showed remarkable efficacy, with the diameter of the induced

inhibition zones ranging from 22 mm to 40 mm, with an average of 31.12 ± 5.38 mm. A good antifungal action was found in both Miconazole and Nistatin.

Miconazole determined an inhibition area with an average of 19.12 ± 1.92 mm, with diameters ranging from 16 mm to 22 mm. Out of a total of 16 strains, 7 were sensitive to this substance and 9 strains were intermediate sensitive.

Nystatin determined an inhibition zone average of 15.19-15.18 ± 3.37 mm, but out of a total of 16 strains, only 3 were sensitive to this substance. The other 13 strains *of M. canis* proved to be intermediate sensitive.

However, analyzing in detail, the sensitivity of each strain was found that all 16 were sensitive to Clotrimazole, but the sensitivity to the other two antifungals (Miconazole and Nistatin) was different, in the sense that there were strains that were sensitive to Miconazole (7 strains), but intermediate sensitive to Nistatin.

Compared to Ketoconazole and Amphotericin B all tested strains can be considered resistant. The diameter of the zones of inhibition induced by the two antifungals was non-existent (microcompet diameter = 5 mm) or extremely small, averaging 6.5 ± 1.88 mm for Ketoconazole and 5.56 ± 0.96 mm for Amphotericin B.

From the literature, the tested substances are known how to act and it is also known the group of fungi on which they act predominantly. Miconazole is an imidazole that was introduced into therapy at about the same time as Clotrimazole. It is mainly used in local applications on dermatophyte-induced lesions in dogs and cats (lotion with Miconazole nitrate 1%) and in the treatment of otitis in carnivores (otic solutions with miconazole nitrate 23 mg / ml). Miconazole has fungistatic or fungicidal action at high concentrations and acts by inhibiting the biosynthesis of ergosterol, which is the essential component of the fungal cell membrane, thus causing a change in its permeability (10).

Ketoconazole is an imidazole compound recommended for the fungicidal and fungistatic effect on dermatophytes, yeasts and certain species of filamentous fungi. At oral administration of 400 mg of Ketoconazole, in dogs, absorption is very good due to the acidic environment in the stomach, but availability for absorbtion varies from 4 to 89%, and long-term treatments have been accompanied by clinical side effects due to toxicity (10).

Ketoconazole has a relatively broad spectrum of action against dermatophytes, yeasts and some species of filamentous fungi, especially those of the genus Aspergillus (10). Ketoconazole is used in veterinary medicine in dogs, cats and other small species for local treatments or baths, but also in the therapy of primary mycoses. There are no specific veterinary products, but products for human use are recommended: Ketoconazole 200 mg tablets and Ketofungol - shampoo with 2% ketoconazole (10).

Clotrimazole is also a derivative of imidazole. It acts fungistatically and / or fungicidal against some species of dermatophytes, against yeasts and against dimorphic fungi (10). Clotrimazole acts by interacting with 14-α demethylase and blocking the conversion of lanosterol to ergosterol, the predominant component of the fungal membrane. It is recommended in topical applications, Clotrimazole 1% cream, being commercially available only preparations for human use (10).

Amphotericin B is a polyenic macrolide antifungal, being very effective especially against yeasts of the genus Candida but also against some pathogenic fungi. It acts by irreversibly binding to ergosterol in the membrane of fungal cells forming membrane pores that allow the loss of ions (K +, Ca2 + and PO43-). Due to its ability to bind to cholesterol present in the cell membranes of higher organisms, it has an important toxic potential (10).

Amphotericin B is usually indicated in humans in some severe systemic fungal infections, when treatment with other antifungal substances does not work, due to the installation of their resistance to the low spectrum of action. In veterinary medicine, Amphotericin B is used in dogs, but has also been used in other species such as cats, horses, llamas and birds. In animals with nephropathy it is contraindicated or used provided that renal function is monitored. There are no veterinary products based on Amphotericin B, but those for human use, injectable products with different concentrations, as well as products for topical applications can be used (10).

Nystatin is a polyene antifungal that has a mechanism of action similar to that of amphotericin B. In veterinary medicine, Nystatin is used in dogs, cats and birds for gastrointestinal fungal infections. As there is no conditioned products for veterinary use, for animal are prescribed products for human use, especially the product Nystatin-oral suspension with 100 000 units /ml (10).

It is known from the literature that, although *M. canis* infection has always been relatively simple to treat with antifungal agents, there are still, in human medicine, patients with recalcitrant infection, whose number is increasing (7, 9). Resistance to treatment with an antifungal substance is considered to be clinical ezpresed when in a clinical case there is a persistent infection or recurrences that reappear 4 weeks after stopping treatment, at a correct dosage of that antifungal drug (5).

Moreover, in the results published in the literature there is no uniformity in terms of testing the sensitivity to antifungal substances of the species *Microsporum canis*. In a recent study realised by Aneke et al. (3) for *M. canis*, high values of the minimum inhibitory concentration for Floconazole were found, using the method of micro-dilution in broth and a reduced inhibition of this antifungal in case of diffusion in agar method. Similar aspects, increasing the resistance to Floconazole of *Microsporum canis* strains have been previously reported by other authors (2, 15).

High values of the minimum inhibitory concentration and low diameters of inhibition zones have been reported for Griseofulvin, using both the broth micro-dilution method and the agar diffusion method (15). Compared to this substance, values greater than 3 µg / mL (for the minimum inhibitory concentration) and a diameter of the inhibition area of less than 16 mm (25 pg / disc) were considered in *M. canis* strains from people indicate an installation of resistance. As these values are considered a limitation of the efficacy of therapy for *T. rubrum*, it is recommended that Griseofulvina not be the first choice for the treatment *of M. canis* infection in both humans and animals (3).

Extensive research, conducted by Afshari et al. (1) on different strains of dermatophyte species, evaluated the antifungal sensitivity to commonly used antifungal drugs using standard fluconazole discs (6.0 mm Dia., MAST Diagnostics) (25 µg), itraconazole (10 µg), 30 mg terbinafine (30 µg), griseofulvin (25 µg), and ketoconazole (10 µg) on glucose (2%) and methylene blue supplemented Mueller Hinton agar 5 µg / ml). These studies showed that the sensitivity of isolated strains of the genus *Trichophyton,* to ketoconazole was 23 ± 0.35 mm, to itraconazole was 22 ± 0.25 mm, to griseofulvin was 16 ± 0.10 mm, and terbinafine was 14 ± 0.25 mm. In strains of the genus *Microsporum,* the mean sensitivity to ketoconazole was estimated at 22 ± 0.15 mm, for itraconazole it was 23 ± 0.15 mm, for griseofulvin it was 16 ± 0.20 mm, for terbinafine it was ± 0.12 mm, for the genus *Epidermophyton,* the mean sensitivity to ketoconazole was estimated to be 23 ± 0.35 mm, for itraconazole it was 24 ± 0.10 mm, for griseofulvin it was 16 ± 0.10 mm, and for terbinafine was 16 ± 0.23 mm (1). Comparative results of drug sensitivity of different dermatophyte species to the antifungal agents examined indicated that the highest sensitivity to ketoconazole was in *E. floccosum* and *T. mentagrophytes* species, the highest sensitivity to itraconazole was found in *T. rubrum* and *E floccosum*, the highest sensitivity to griseofulvin was found in *T. verrucosum*, and the highest sensitivity to terbinafine was revealed for *T. verrucosum* (1).

Overall, susceptibility to ketoconazole and itraconazole tested in this study was higher than in other types of antifungals, including griseofulvin and terbinafine. However, these results are different from those obtained in our study.

Conclusions

The present study revealed that of the five substances tested for antifungal efficacy on some strains of *Microsporum canis,* Clotrimazole showed outstanding activity, causing areas of inhibition that were well above the sensitivity limit given by the microcab manufacturer.

Miconazole and Nystatin acted with lower efficacy, given smaller inhibition areas smaller than Clotrimazole, from intermediate to sensitive.

Of the total strains tested, all presented sensitivity to Clotrimazole, but sensitivity to the other two antifungals, was different, being observed strains sensitive to Miconazole but intermediate to Nystatin.

In Ketoconazole and Amphotericin B, all tested strains were considered resistant, the area of inhibition being non-existent or very small.

Sensitivity tests for antifungal substances for dermatophytes should become common in veterinary and human practice both to ensure the effectiveness of treatment and to prevent the onset of resistance.

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PROTOSAN INFECTION OF SMALL RUMINANTS IN SOUTH PART OF SERBIA WITH EMPHASIS TO NORTH KOSOVO

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Summary

Enteral protozoan infection was of great importance to health status of small ruminants and its performances. This was parasitic infection caused by protosoas from genus *Eimeria, Cryptosporidium* and *Giardia.* Lamb and kids infection had moderate morbidity and low mortality rate. Clinical sign of infection is usually presented in animals at 4-10 days old. The study about parasitic fauna - protozoa helminths, and arthropods of small ruminant at south part of Serbia, with emphasis to North Kosovo were performed during 2017. The study included the examination in total of herds flocks of goats and sheep from Zvečan and Leposavić district (villages Ceranja, Majdevo, Zemanica, Mure, Rudine, Žitkovac, Oraovica, Mošnica, Donji Krnjin, Belo brdo, Mioliće, Drenova and Beliće. Colected faeces samples were examined using routine coprological methods. Determination of parasites we performed by morphological characteristc. Infection with protosoa occurred at and on 46.14% of examined sheep and 29.42% of examined goat herds. Coccidiosis was found at 43 sheep and 27 goat herds. We usally occured mixed infection with 2-3 coccidia species. At sheep most abundant species were *E faurei*, followed by *Eimeria ahsata*, *E.ovinoidalis, E. intricata* and *E. pallida*. At goats most abundant species were *E.arlongy*, folwed by infection with *E. hirci*, *E.nina-kohl-yakimovae*, *E. christenseni* and *E. caprina*. Oocyst were found at adult ant young animals, but clinical sign of disease were present only at young animals. During our examination *Cryptospoidium spp.* was found at 29 sheep and 23 goat herds. Determination of subspecies we not performed. Lambs between five and twenty-one days were the most susceptible for infection. Lambs cryptosporidiosis has high morbidity and mortality rate. Symptoms of acute cryptosporidiosis include inapetence, and weight loss. Infection with *Giardia duodenalis* was found at 2 sheep herd. Determination of subspecies we not performed.

Keywords: North Kosovo, Serbia, small ruminants, protozoa infection

Enteral protozoan infection was of great importance to health status of small ruminants and its performances. This was parasitic infection caused by protosoas from genus *Eimeria, Cryptosporidium* and *Giardia.* Infections are worldwide present and it is nearly impossible to find a flock without some protoza infection (2, 14, 24, 34, 37, 45). Usually poor management is the reason why numbers of protozoan infection increase excessively; thus, coccidiosis may be considered a man-made

disease (1, 11, 30, 48). This also suggests that protozoosis can not be adequately managed (5, 7, 11, 18, 26). Usually it is presented at young animals at 4-10 weeks. Infection had moderate morbidity and low mortality rate. Environmental contamination and resulting clinical disease is generally influenced by local weather conditions and the grazing management practices of the flock (10, 21, 42).

The economic impact of protzoan infection in small ruminants is not well documented and there is no published data about estimate for economic losses due to subclinical or clinical disease. The economic cost is considerable, in terms such as low growth performance, decrease in productivity, mortality, morbidity, and the cost of prevention and treatment (4, 23, 32, 38, 39).

Protozoan infection in small ruminants (sheep and goats) in Serbia has been examined in the last fifteen years and in our paper we presented results of the our examination at south part of Serbia, with emphasis to North Kosovo (the status of Kosovo is in accordance with UNSCR 1244 and the Opinion of the International Court of Justice on the Kosovo Declaration of Independence).

Materials and methods

During 2017 we examined 114 herds of small ruminants from Zvečan and Leposavić district (villages Ceranja, Majdevo, Zemanica, Mure, Rudine, Žitkovac, Oraovica, Mošnica, Donji Krnjin, Belo brdo, Miolićе, Drenova and Belićе). Geographical conditions in examined area favor for breeding of small ruminants. In this area is mostly mountainous and hilly, with large areas under pastures (31). All herds were examined for the presence of ticks, gastrointestinal and pulmonary helminths and protozoa (30, 44).

During study we collected fecal samples during the whole year. Grazing animals of both sexes (220 males and 380 females, a total of 600) were randomly chosen. There were 410 adults (one-year-old and above) and 190 lambs and kids. Coprological examinations we performed with faecal concentration techniques, especially zinc sulphate flotation, and with sedimentation technique (40, 49, 50). Direct smear or wet mount examination for oocyst and trophozoites can also be performed. However, because of the cyclical nature of cyst excretion, several samples need to be examined to detect the organism.The diagnosis of parasites is commonly established by microscopic identification of oocyst, cysts or less commonly trophozoites in faecal wet smear stained with iodine (9, 24). Determination of subspecies of cryptosporidia and giardia we not performed. Examinations we performed with AxioLab A1 microscope with the Axiocam 105 Color microscope camera and Zen Lite software, manufactured by Carl Zeiss.

Results and discussions

Infections with protosoa occurred at and on 46.14% of examined sheep and 29.42% of examined goat herds.

Coccidiosis were found at 43 sheep and 27 goats herds. We usally occured mixed infection with 2-3 coccidia species. At sheep most abundant species were *E faurei*, followed by *Eimeria ahsata*, *E.ovinoidalis, E. intricata* and *E. pallida*. At goats most abundant species were *E.arlongy*, folwed by infection with *E. hirci*, *E.nina-kohl-yakimovae*, *E. christenseni* and *E. caprina.* Oocyst were found at adult and young animals, but clinical sign of disease were present only at young animals (2, 13, 14).

During our examination *Cryptospoidium spp.* was found at 29 sheep and 23 goat herds. Symptoms of acute cryptosporidiosis include inapetence, weight loss, and diarrhea which is usually yellow to yellowish-brown and of a creamy texture (23). The rapid loss of nutrients and fluids during diarrhea results in dehydration. Some animals do not develop into chronic cases and become continuous carriers of infection (13, 14, 15, 17).

Infection with *Giardia duodenalis* was found at 3 sheep herds.

During our examination we established that usually poor management is the main reason why numbers of protosoan infection increase excessively; thus, may be considered by adequately managed (5, 7, 11, 18, 26). The parasite causing infection is passed through fecal to oral contact. Adult animals were main source of infection, because they permanent excreted oocyst by faeces (29, 38, 42). Presence of oocyst in stables induced contamination of food and water and infection to young animals.

Parasites of the genus Eimeria cause a disease commonly called coccidiosis. Coccidiosis is known as a "stealth killer" of goats because symptoms are easy to miss and irreversible damage can be done if the illness is not quickly treated (9, 10, 30). Historically, some *Eimeria* spp. were thought to be infectious and transmissible between sheep and goats, but the parasites are now considered host-specific (29). At sheep were established next coccidial species: *Eimeria ahsata*, *E. ammonis, E. arkhari, E. crandallis*, *E. dalli, E. danielle*, *E. faurei*, *E. gilruthi*, *E. gonzalezi*, *E. granulosa, E. intricata, E. marsica*, *E. ovina*, *E. ovinoidalis*, *E. pallida*, *E. parva* Kotlán, *E. punctata* and *E. rachmatullinae* (14, 15). At goats were established *Eimeria absheronae, E. africiensis, E. alijevi, E. arloingi, E. babaevi, E. caprovina, E. christenseni, E. hirci, E. jolchijevi, E. kocharii,* and *E. nina-kohl-yakimovae* (9, 21, 23, 24, 38). All species of coccidia are not disease causing. There are only some species that are responsible for the outbreak of the disease.

For sheep *E. ovinoidalis* can be very pathogen and other species such as *E. bakuensis* (*E. ovina*) and *E. crandallis* may exacerbate the symptoms of the former two species. The most pathogen Eimeria species for goats are *E. nina-kohl-yakimovae,* followed by *E. arloingi* and *E. christenseni* (20, 30). This was confirmed during our research both in the north of Kosovo and in other areas of Serbia (37, 38, 41, 42).

Signs of clinical disease we generally occur about 18 to 20 days after ingestion of sufficient amounts of coccidia oocysts from the contaminated environment. Clinical coccidiosis occurs when damage to the gut is sufficiently severe to cause dysfunction. This normally occurs at the beginning of the parasite's sexual multiplication stage, when parasite numbers reach their peak (48). Due to the

damage of the cells lining the intestines, the primary symptoms of coccidiosis is diarrhea, which may be foul smelling and contain mucus and blood. Diarrhea may have a dark tarry appearance and, in severe cases, large blood clots can be seen (22, 20, 52).

At small ruminants were established severall *Cryptosporidium* species: *C. parvum, C. hominis* (previously *C. parvum* genotype 1). *C. canis, C. felis*, *C. meleagridis,* and *C. muris*. (18, 45, 46). This is parasitic disease with clinical signs at lambs and kids old at 4 to 10 days (27, 28, 35). During our examination we established that animals between five and twenty-one days were the most susceptible for infection Cryptosporidiosis has high morbidity and mortality rate. Symptoms of acute cryptosporidiosis include diarrhea, inapetence, and weight loss (44, 45, 48). The rapid loss of nutrients and fluids during diarrhea results in dehydration. Since intestinal tract cells are disrupted, absorption of feed nutrients is restricted, and the animal loses more nutrients through the digestive tract and have lover feed conversion ratio (7, 11, 51).

During our research diarrhea which is usually yellow to yellowish-brown and of a creamy texture especially on the second and third day from the onset of clinical symptoms (35, 36).

G. duodenalis are a flagellate parasite, is one of the most prevalent and widespread intestinal parasite in humans and numerous vertebrate animal (mammals, birds, amphibian). Infection are spread worldwide (3, 8). Parasites causing a diarrheal condition known as giardiasis. According morphology and genetic evidence six species have been recognized in the genus *Giardia* but only *G.duodenalis* caused infection of small ruminant. Phylogenetic analysis and enzyme electrophoresis examination *G. duodenalis* revealed the existence of eight assemblages A–H within the species. In goats higher occurrence genotype E, with genotypes A and B being less frequent (6, 16, 19, 25). Ruminants which infected with *G. duodenalis* are mostly asymptomatic, but subclinical signs such as impairment in feed conversion efficiency, reduction in growth rate and persistent diarrhea. During our examination we confirmed that giardiasis is more surveys from sheep than goat populations. This is also indicated by the fact that it is fewer publications on giardia in goats (3, 12, 13, 30, 47, 53).

During this examination was first time established giardiasis in sheep in Serbia. Later, during 2018, we established first occurence of giardiasis in goats breeding in south part of Serbia near by north Kosovo (43). Our research confirmed the presence of *Giardia duodenalis* in small ruminants herds in Serbia.

Conclusions

Enteral protozoan infection was of great importance to health status of small ruminants and its performances. This was parasitic infection caused by protosoas from genus *Eimeria, Cryptosporidium* and *Giardia.* Infection usually had moderate morbidity and low mortality rate. Consequence is significant increase of lambs and kid accrescense, its weakens and less growth. The best preventive measure a sheep and goat producer can take is to use a feed with a coccidiostat added. With careful management and sound preventive measures, the losses associated with this disease can be reduced to minimal level.

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CONSIDERATIONS ON THE USE OF *ROCURONIUM* IN OPHTHALMIC PRACTICE IN DOGS

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Summary

In ophthalmology, muscle relaxants are used to maintain the central position of the eye ball and immobilize it. Because dependent of the dose determines the decrease of skeletal muscle contraction to paralysis, manual or mechanical ventilation becomes mandatory. Recommended dose of rocuronium for endotracheal intubation in dogs is 0.3-0.6 mg/kg. The effects of rocuronium on the extraocular muscles could also be observed at doses used in the present study: 0.03 mg/kg and 0.16 mg/kg, providing conditions for an ophthalmological exam, respectively for surgeries lasting up to 27 minutes. The second used dose, administered in fractions at intervals of 3 and 5 minutes respectively under injectable/inhalation anesthesia may impair respiratory dynamics.

Keywords: rocuronium, ophthalmology, dog

Muscle relaxation means paralysis of the skeletal muscles in order to facilitate maneuvers and techniques related to anesthesia or surgery. In certain circumstances, this optional component of general anesthesia becomes mandatory.

The general indications and advantages of the use of these drugs in veterinary practice are:

- relaxation of skeletal muscles in order to facilitate surgical access without compromising the recovery from anesthesia: In fact, this is the main purpose for which they are used. They not only assure an easier access, but their use minimizes the appearance of bruises due to the use of spacers, thus contributing to post-operative comfort and faster healing of the patient. They are very useful in long-term interventions that require absolute relaxation, such as intraocular procedures or microsurgery. In ophthalmological operations, they are used to maintain the central position of the eye and immobilize it. In most species, extraocular muscle relaxation is easy to be obtained and small doses of medication are used. In the case of surgeries performed on the eyeball one of the requirements is to keep it immobile in a central position (10, 16). In pets in stage of surgical anesthesia, the position of the globe changes by rotating to the nasal angle exposing the sclera (10). This position is not an ideal one for intraocular or corneal surgeries. The use of non-depolarizing muscle relaxants in order to relax the extraocular muscles allows the eye to remain in a central position that clearly facilitates the surgical intervention. They also are

used in interventions that require specific muscle relaxation: median celiotomy, cesarean section, diaphragm surgery (repair of diaphragmatic defects) (15, 16).

The use of muscle relaxants reduces the dose of general anesthetics and thus protects cardiovascular function and reduces the duration of surgery in patients at high operative risk (15).

- to facilitate breathing in intrathoracic interventions but also to assist poor breathing, for example in dogs with bradypnea (5).
- when aiming to suppress unwanted spinal reflexes that may occur during general anesthesia, for example head movements during ear surgery (15).
- assistance in treatment of joint dislocations, bone fractures. Treatment of fractures/dislocations is not easy due to the spastic contraction of the regional muscles caused by hematomas or bone fragments resulting from the fracture.
- to facilitate airway instrumentation, namely, endotracheal intubation and endotracheal endoscopy. Although most animals can be intubated without muscle relaxants, they can make intubation much easier in cats and pigs (5).

There are several groups of drugs that that provides muscle relaxation. These are represented by the nonspecific medication, that contributes to muscle relaxation and which is represented by local analgesics (the area served by the blocked nerve loses its muscle tone) and by centrally acting drugs (general anesthetics, phenothiazine derivatives, benzodiazepine tranquilizers, alfa-2 adrenergic agonists). There is also a specific relaxant medication that includes central muscle relaxing drugs (that act on the spinal cord blocking nerve impulse transmission) and peripheral muscle relaxants (depolarizing and non-depolarizing that act on the neuro-muscular junction of striated muscles) (20).

Non-depolarizing muscle relaxants block specific acetylcholine receptors (competitive inhibition) without intrinsic effect. The neuro-muscular plate remains polarized, at rest, resulting a polarization block (14). To observe an effect at least 75% of the receptors must be occupied by non-depolarizing relaxant molecules, and up to 92% for complete neuromuscular transmission block (8).

Most of the non-depolarizing agents have benzylisoquinoline or steroidal structure (1, 6, 8). Rocuronium belongs to the category of aminosteroids or pahicurars (round fat molecules) (8).

The criteria underlying the choice of a particular muscle relaxant are numerous, the choice is made according to:

- anesthetic and surgical requirements;
- speed of muscle relaxation installation;
- duration of action required;
- possible side effects (8).

Because it causes a dose-dependent decrease in the contraction force of the skeletal muscles to paralysis, manual or mechanical ventilation becomes mandatory after the administration of peripheral muscle relaxants (11, 16). Mechanical ventilation allows the anesthetist to perform other duties, ensures a constant volume and respiratory rate and is therefore preferable (16). Unfortunately,

the ventilators are missing from the endowment of the vast majority of our veterinary offices.

In this paper work, we aimed several objectives, to identify the minimum dose that ensures optimal working conditions for ophthalmological investigations and eye surgery when using rocuronium and last but not least, if it is possible at the same time to avoid ventilatory support.

Materials and methods

In a number of 40 dogs, clinical cases, classified in anesthetic risk class I or II, after the installation of the surgical anesthesia plane, it was decided to complete the anesthetic formula randomly using the non-depolarizing muscle relaxant rocuronium (Esmeron 1%) administered by IV , as follows:

- in first group, G1 (n=10), for premedication acepromazine (Calmivet 0.5%) was used and for induction and maintenance propofol (Lipuro 1%). Patients were intubated and connected to the anesthesia machine to improve oxygenation by administering oxygen and to support ventilatory function if required by manual ventilation. A single dose of 0.03 mg/kg rocuronium was administered.
- in G2 group (n=10) induction and anesthesia were performed by inhalation with isoflurane (Anesteran), after a premedication with acepromazine (Calmivet 0.5%) and ketamine (Ketamine 10%). A single dose of 0.03 mg/kg rocuronium was administered;
- in G3 group (n=10) acepromazine was used in premedication and for induction and maintenance propofol. All patients were intubated. An initial dose of 0.1 mg/kg followed by twice administrations of 0.03 mg/kg at intervals of 3 minutes after the initial dose;
- in G4 group (n=10) the anesthetic protocol was identically with those of G2 but repetition of rocuronium in a dose of 0.03 mg/kg, after the initial dose of 0.1 mg/kg, was performed two times at 5-minute intervals.

In all cases, monitoring of neuromuscular block was performed subjectively. Thus, the following was followed:

- the time interval in which the muscle relaxant effect appears by returning the eyeball to the central position;
- the duration of its maintenance in a central position;
- the time elapsed until the reappearance of spontaneous respiratory movements;
- the interval in which patients' breathing was controlled manually/mechanically, in other words the moment when spontaneous respiratory movements were considered to be effective in terms of amplitude and frequency.

The data obtained were statistically processed, being expressed by arithmetic mean and standard deviation, and for comparisons between groups the t-Student test was used, considering significant differences for values of $p \leq 0.05$.

Results and discussions

The patients belonged to both sexes (15 males and 25 females), were both puppies and adults aged between 4 months and 15 years and belonged to small, medium or large breeds, or their half-breeds.

The recommended doses of rocuronium for endotracheal intubation in dogs are 0.3-0.6 mg/kg (2, 5). Doses ten to twice as low (0.03 and 0.16 mg/kg) as the minimum dose were used in the present study. The use of these low doses is motivated firstly by the lack of equipment for monitoring the intensity of the neuromuscular block (by mechanomyography, electromyography, acceleromyography, measuring the electrical response evoked by a piezoelectric sensor, phonomyography), the monitoring performed by us being subjective, necessary to identify the block residual and secondly because our goal was not that to obtain myorelaxation to facilitate endotracheal intubation.

A study conducted in 2018 (17) indicates that 43% of the 390 American, Spanish and Brazilian veterinarians surveyed, monitor and subjectively assess neuromuscular function after the use of muscle relaxants, by observing spontaneous movements, including spontaneous breathing. Recovery from the muscle relaxant effect is also subjectively assessed by 35% of respondents. Therefore, the choice of the use of muscle relaxants in the present study in the conditions of a subjective monitoring implies the assumption of a calculated risk in the conditions in which at any moment if necessary the support of the ventilatory function of the patient can be realized. Another aspect that the previous study highlights is the fact that only 18% of participants use residual block reversal agents, therefore a very small number, which justifies the longer monitoring of each patient during the return from anesthesia. The natural conclusion of the study is that there is a limitation in the implementation of monitoring techniques, criteria for repeating the administration of muscle relaxant, the use of reversing agents and monitoring the recovery of neuromuscular function suggested by scientific research (17).

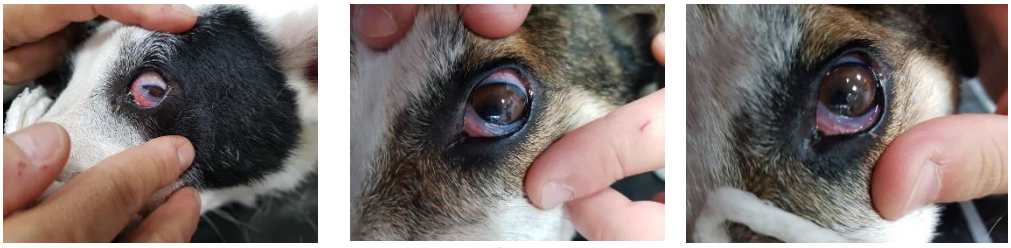
Secondly, dose reduction, which was an advantage in our working conditions, was possible because it is known that extraocular muscles are very sensitive to neuromuscular block (19). The onset of neuromuscular block is faster in the central muscles with a good blood supply, for example diaphragm and larynx. Conversely, peripheral muscles with a relatively low blood supply will have a slower onset of the block and a longer recovery, for example the thumb adductor. The anterior airway muscles and the pharynx behave like the central muscles at the beginning, they are sensitive to the effect of muscle relaxants but the recovery is slow as in the case of peripheral muscles (18). The orbicularis muscle of the eye is probably the ideal muscle to be monitored at the time of induction and intubation because it is very similar to a central muscle, and will mirror the onset of block in the larynx and mainly the diaphragm (7).

Therefore, the effects of muscle relaxant on the extraocular muscles, muscles considered central, could be observed in the conditions of the doses used by us in this study.

After the administration of rocuronium to groups G1 and G2 we found the following:

- rotation of the eyeball returning to the central position in a time interval with limits between 3-18" in group G1 and 2-9" in group G2;

- the central position of the eyeball (Fig. 1) with the iris fully visible is maintained 140±21 "in group G1 and 293±25" in group G2. The differences between the two groups are statistically significant ($p=1.45x10^{-10}$) (Fig. 2). The longer time obtained in group G2, double the average of that in group G1, can be explained by the potentiation of the muscle relaxant effect by the inhaling narcotic.



a b c

Fig. 1. Eyeballs position, patient group G1, a - plan of surgical anesthesia, eyeball rotated to the nasal angle, b and c - surgical anesthesia, at 5" and 15" respectively after administration of the calculated dose of muscle relaxant

The obtained times are not long enough for surgeries but allow short-term examinations of the structures of the eyeball. The data obtained in the present study in group G2 can be compared with those obtained by Briganti et al. (4), in which the maintenance of the eye in a central position at a dose of 0.03 mg/kg rocuronium has a duration of 8.7 ± 2.8 minutes. The lower values from the G2 group can be attributed to the different anesthetic formula, in our case no alpha-2 adrenergic agonist was introduced in the anesthetic formula, as used in the above mentioned study. It is known that dexmedetomidine in addition to its sedative effects also has muscle relaxant properties that are synergistic with those of isoflurane and of course with rocuronium;

- in all patients belonging to both groups the pupil remained in miosis;

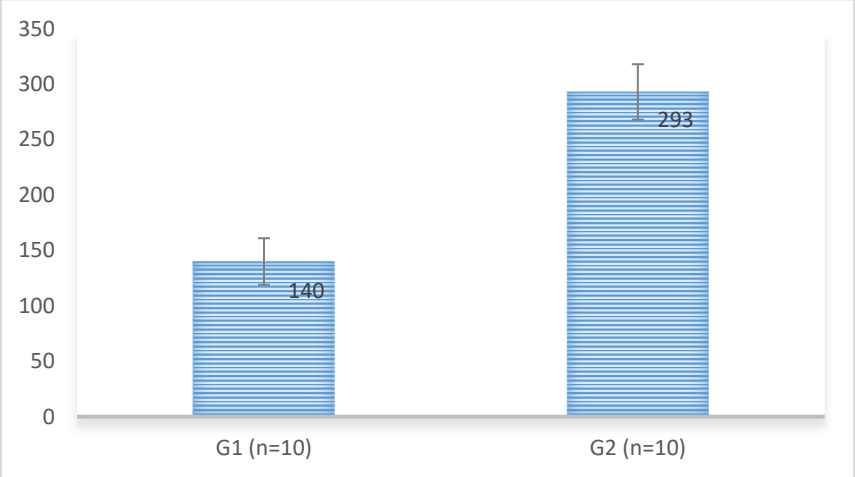


Fig. 2. Representation of duration (seconds), mean values ± standard deviation, central positioning of the eyeballs in individuals of groups G1 and G2

- spontaneous respiratory movements were maintained in all patients of both groups, they had amplitude and frequency considered normal (13, 21, 22). In both groups the current volume was between 10-20 ml/kg, the respiratory rate had limits between 10-25 breaths/minute, capnography indicated values between 35-43 mmHg, and pulse oximetry hemoglobin saturation values of 93-100%. These data confirm that oxygenation is as effective as the elimination of carbon dioxide, therefore ventilatory function and respiration are not negatively affected by muscle relaxant.
- no statistically significant differences were found between the two groups in terms of blood pressure, even if the mean values of systolic, diastolic and mean blood pressure recorded in group G2 were lower than in group G1. In both groups there was a decreasing tendency of tension but with maintaining within normal limits under anesthesia (22). Our data do not confirm the data obtained in another study in which the transient increase in blood pressure was found (9) but in that study the dose of rocuronium was 0.4 mg/kg, so much higher than the one used by us. Taking into account the fact that the values of heart rate, another parameter monitored, did not have large oscillations within normal range, ie did not fall below 70 and did not exceed 124 beats/minute, we consider that the data obtained by us confirms the statement of Grimm et al. (12), who claim that rocuronium does not induce cardiovascular changes.

In group G3, 0.12 mg/kg rocuronium was administered within six minutes and the following were observed:

- in animals under surgical anesthesia with the eyeball rotated mid-ventral to the inner corner of the eye, after administration of the loading dose according to the principle of priming, the eyeball returns to the central position within a few

seconds, without exceeding the limits recorded in group G1. Positioning towards the nasal angle of the eyeball during anesthesia is the result of uneven relaxation of the extraocular muscles (10) and returning to the central position denotes the installation of the effect of rocuronium. The time in this study is much shorter (3-18") than in the literature (45 ± 7") in a study (3) in which animals were anesthetized with medetomidine-methadone-propofol and rocuronium was administered in a single dose of 0.1 mg/kg, a dose similar to that used by us;

- repeated administration of muscle relaxant allowed the eye to be kept in a central position for a period of time with limits between 14 and 17 minutes, an interval comparable to that reported in other studies (5, 6). This time allows minor surgery;

- regarding the monitored functional parameters, we found only in 2 patients the deterioration of the respiratory parameters, ie the decrease of the current volume to values of 5-8 ml/kg, the increase of $EtCO_2$ to values of 47-55 mmHg, the reduction of the respiratory frequency to 6-11 breaths/minute, the installation of bradycardia and the reduction of TAM values by up to 9% compared to the initial values, which required the establishment of manually assisted ventilation with increasing amplitude and frequency until the normalization of the parameters listed above. Deterioration of these parameters was found in one patient after the first dose of rocuronium and in another immediately after the third dose. The ventilation assistance lasted 10 minutes in the first case and 15 minutes in the second. In the other patients included in the study, the values of the functional parameters were maintained within normal limits with spontaneous breathing throughout the anesthesia, implicitly of the cure.

In group G4, 0.12 mg/kg rocuronium was administered within 10 minutes and the following were observed:

- all patients breathed spontaneously during treatment and anesthesia. Similar data were obtained in a study in which the dose of muscle relaxant used was 0.1 mg/kg but in a single administration (3);

- on the whole, the ventilatory functional parameters were kept within limits considered normal, with transient oscillations in six of the patients outside them, during which time it was intervened with assisted ventilation and increased oxygen flow, but not more than 7-13 minutes;

- the hemodynamic functional parameters varied in the sense of reducing the TAS by up to 13%, the TAD by up to 15% and the TAM by up to 15% compared to the initial values;

- after administration of the first dose of muscle relaxant, the eyeball returned to its central position within a few seconds, as was found in group G2. The time interval in which the position was maintained extended from the first administration for 20-27 minutes. The small doses of muscle relaxant used and administered repeatedly in the case of inhaled anesthesia are effective in terms of the purpose of this paper and the explanation is partly due to the potentiating effect produced by the gaseous anesthetic. The lack of cumulative effect and short dose-related duration of action were compensated by repeated administration. In fact, the literature mentions that it can be used in long-lasting infusions (8).

- after the administration of the last dose of rocuronium, the change in pupillary diameter was observed in all patients with the installation of a mydriasis, sometimes extreme, which lasted for 20-30 minutes.

This common phenomenon after topical administration of iridocycloplegic anticholinergic mydriatics, sympathomimetic mydriasis, alpha-2 adrenergic agonists or opioids (12) is to some extent difficult to explain in the case of muscle relaxants whose effect is manifested only on striated muscles. The iris muscles, constrictor and dilator, are smooth multiunit muscles organized in motor units similar to those of striated muscles, each muscle fiber receiving individual motor innervation. The excitability of these muscles is lower than the striated ones and the excitation of the smooth muscle fibers is done by membrane depolarizing mechanisms or by hormonal mechanisms, with the involvement of secondary messengers of AMP-cyclic, GMP-cyclic type, without membrane depolarizations. Smooth muscle contraction can be triggered or inhibited by local factors (CO_2, O_2, lactic acid, etc.) or circulating hormones (norepinephrine, adrenaline, serotonin, prostaglandins), including histamine (23). Based on the information that rocuronium causes the release of histamine (8), it can be hypothesized that this, combined with the influence of other factors, including a deep anesthetic plan, causes the changes we observe. This mydriasis on the one hand would be favorable for intraocular surgery, lensectomy for example or cataract surgery by phacoemulsion with anterior capsuloresis, but makes it difficult to monitor the depth of anesthesia dynamic pupillary being an important indicator of it and signaling the installation of too deep anesthetic or pain.

Conclusions

The effects of rocuronium on the extraocular muscles could also be observed at doses and conditions of the present study (0.03 mg/kg and 0.16 mg/kg).

The dose of 0.16 mg/kg of rocuronium administered in fractions at five-minute intervals allows the duration of the central eye to be extended to 20-27 minutes under inhalation anesthesia.

Deterioration of respiratory dynamics, without the onset of apnea, has been reported in some cases after dose of 0.16 mg/kg and has been corrected by assisting respiration and increasing oxygen flow.

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VANCOMYCIN - RESISTANT *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* SCREENING AND ISOLATION FROM UTERINE DISCHARGE OF DAIRY CATTLE IN THE FIRST FIVE WEEKS POSTPARTUM

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Summary

Enterococcus spp. is a commensal of natural gut microflora in bovine and human. The main objective of the research was to characterize the uterine bacterial flora of dairy cows diagnosed with puerperal endometritis and to identify pathogenic bacterial species associated with uterine diseases. The isolates of enterococci were cultured from uterine discharges. For direct identification of VRE strains chromogenic medium was used. Given the pathogenic potential of enterococci and their ability to develop antibiotic resistance with direct consequences on antibiotic therapy, we set out to screen for *Enterococcus fecalis* and *Enterococcus faecium*, the most isolated species of enterococci in the microbiota of endometrial secretions. Samples were collected from 18 cows in the first five weeks postpartum. The prevalence of *Enterococcus faecium* in pure culture was highest in fourth and fifth week after parturition, when rate was 16.66 % and for *Enterococcus faecalis* the highest prevalence was in the first and second week, when rates were 16.66% and 11.11%, respectively. A consequence of the use of antibiotics in cattle production systems is the development of antimicrobial resistance. Resistance to penicillin, tetracycline, streptomycin, and gentamicin was frequent among the isolates. The fact that enterococci are recognized for their role as pathogenic opportunists and for their natural and acquired resistance in human pathology and to the same extent, they are isolated from animals, we can support the hypothesis of a cross-infection between humans and animals.

Keywords: Dairy cattle, Uterine discharge, VRE strains, Antimicrobial resistance

Uterine infections following the postpartum period are found daily on dairy farms and are one of the main causes of animal slaughter (22, 26). The most common uterine diseases in dairy cows are puerperal metritis and clinical endometritis (24).

These genital pathologies are due to the inability of the body to eliminate pathogens from the uterus, an inability derived from the intervention of risk factors, such as twin gestation, placental retention, ketosis, hypocalcemia (11, 24, 31).

Most cows eliminate the uterine bacterial flora in the first five weeks after parturition, but this is influenced by a suitable uterine environment, genetic factors and the animal's innate and acquired immunity (12).

The diagnosis of endometritis is based on the presence of vaginal discharges, but the confirmation of the disease is given by the microbiological examination of the tampons collected from the uterine lumen (8, 15, 30).

Bacterial infections that occur in dairy cows are treated with antimicrobial drugs. Most often antimicrobial therapy is essential for the treatment of sick animals, but the application of treatments without microbiological analysis and antibiogram leads to the emergence of antimicrobial resistance (1).

In the context of clinical endometritis, recent studies have identified among the most commonly isolated intrauterine bacteria potential or opportunistic pathogens, including members of the genera *Streptococcus* and *Enterococcus*, in addition to *Staphylococci* or *Bacillus* (32).

Enterococci are commensal bacteria in the intestines of humans and animals, but can cause infections in humans. They can survive and live in hostile environments and are therefore difficult to eradicate in both animal production and clinical settings (16).

For many years enterococci were considered harmless to humans, but more and more studies have reported that they are one of the most common causes of surgical wound infections and urinary tract infections and the third most pathogenic cause of bacteremia (18, 27).

Infection with vancomycin-resistant strains of *Enterococcus faecalis* and *Enterococcus faecium* is often critical because it can lead to urinary tract disorders, endocarditis, and wound infection (9, 23).

Enterococcus spp. are resistant to antimicrobials including cephalosporins, β-lactams and aminoglycosides (33).

Clinical and animal *Enterococcus* isolates with multi-drug resistance to macrolides, tetracyclines, streptogramins, and glycopeptides have also been described (14, 16). In the animals, antibiotic resistance and difficulties in treating enterococcal infections have occurred due to the use of antimicrobial substances as growth promoters (7).

The present study aimed to identify and screen the enterococci present in the uterus and determining the susceptibility of enterococci to recommended antibiotics in bovine endometritis.

Materials and methods

A total of 90 samples were collected from dairy cattle in the first five weeks after parturition. The uterine samples were obtained from dairy cattle in two commercial farms of county Iași. Uterine samples were collected with a sterile cotton swab by wiping the external cervical ostium. Samples were kept and transported to the laboratory in less than 24 hours after collection.

The presence of *Enterococcus* was determined by direct identification on selective medium for the detection of VRE strains, VRE Select™ Agar from Bio-Rad Laboratories. After 24h of incubation at 37°C, the appearance of pink colonies

confirmed the presence of *Enterococcus faecium* and blue colonies the presence of *Enterococcus faecalis* (Fig. 1).

Enterococcus species specific colonies that have developed on the chromogenic agar were transplanted into Columbia CNA+5% Sheep Blood and Bile-Esculin-Azide Agar. Specific colonies of *Enterococcus faecalis* and *Enterococcus faecium* have been identified according to the following criteria: hemolysis on blood agar, their ability to use pyruvate, potential to hydrolyze esculin. For confirmation of *Enterococcal* strains API 20 STREP galleries supplemented with conventional tests: Gram stain and direct catalase test were used.

The antimicrobial sensitivity of the isolates was tested for 9 types of antibiotics and was determined using the disk diffusion method described by NCCLS (2001). The antibiogram was performed on Mueller-Hinton Agar (MHA) and antibiotic sensitivity was determined by measuring the area of inhibition.

Enterococci have intrinsic resistance to various categories of antibiotics, such as cephalosporins and aminoglycosides (at doses used clinically) and aquire through various mechanisms, resistance to penicillins, glycopeptides and aminoglycosides.

The antibiotics selected for antimicrobial testing are part of various groups of antimicrobial substances used in antimicrobial therapy in cattle.

Enterococcal strains were classified as resistant, sensitive and moderately sensitive according to NCCLS criteria (for amoxicillin, colistin, cefquinome, enrofloxacin, marbofloxacin, florfenicol, lincomycin, streptomycin, gentamicin, tetracycline).

Results and discussions

The present study included 18 Black and White Romanian cows and Holstein dairy cows from two farms located in county Iași.

Samples were collected for five weeks from day 3 after calving and up to more than 28 days postpartum.

The collection of mucus from the cervical ostium was done carefully to avoid bacterial contamination, so the cow's vulva was cleaned with dry paper towels, after which a Polanski speculum with three valves was inserted into the vagina. After examining the vaginal walls, the secretions were collected with cotton swabs attached to a metal rod (20, 31).

The type of uterine disease was assessed by observing the appearance and smell of vaginal discharge (10). Starting on day 21 ± 1 and day 28 ± 1 postpartum, the diagnosis of puerperal endometritis was established for 12/18 animals, based on the gynecological examination and the appearance of leaks.

A total of 90 samples were included in the study. The isolation and direct identification of *Enterococcus spp.* was carried out on chromogenic agar plate, VRESelect^{TM}Agar (Bio-Rad Laboratories).

VRE strains, identified as *Enterococcus faecium* and *Enterococcus faecalis*, were isolated from 6 of the 18 cows.

Enterococcus faecalis was isolated in pure culture in cows with endometritis in the first two weeks after parturition in 3 (16.66%), respectively 2 (11.11%) of the samples, and *Enterococcus faecium* in 3 (16.66%) in fourth and fifth week after parturition.

There is a correlation between the category of pathogens identified and the day of sample collection, which highlights a significant increase in the number of females in which *Enterococci* were isolated after 21 days of calving (Table1). This indicates the possibility of recontamination.

Table 1

Incidence of the species *Enterococcus faecalis* and *Enterococcus faecium* isolated from uterine discharges

Tested cows	**18**	**18**	**18**	**18**	**18**
	7 days	14 days	21 days	28 days	>28 days
Enterococcus faecalis	16.66%	11.11%	5.55%	11.11%	11.11%
Enterococcus faecium	0%	5.55%	5.55%	16.66%	16.66%
Enterococcus faecalis+ *Enterococcus faecium*	16.66%	16.66%	11.11%	27.77%	27.77%

To confirm the presence of VRE strains of *Enterococcus faecalis* and *Enterococcus faecium*, isolated colonies from VRESelect™ Agar were inoculated on Bile Esculine Azide Agar (BEA) (Bio-Rad Lab).

Black colonies growing on BEA Agar have been identified as *Enterococcus spp.* (Fig.1). VRE positive strains isolated from BEA medium were confirmed by biochemical tests and Gram staining.

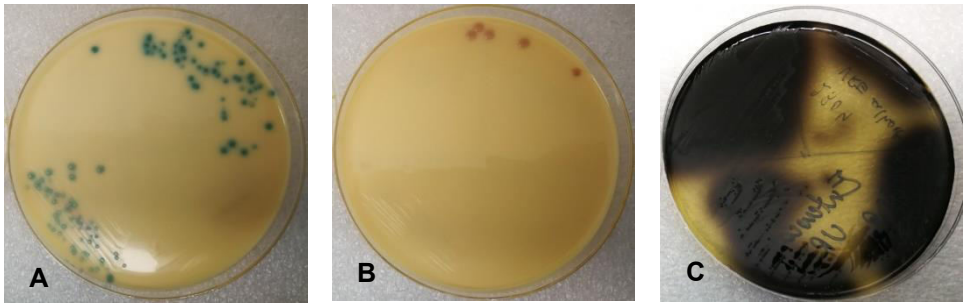

Fig.1. A - *Enterococcus faecalis* (blue colonies)
B - *Enterococcus faecium* (pink colonies) on VRESelect™ Agar
C - Bile esculin azide agar. Black colonies of *Enterococcus*

At more than 21 days postpartum following the diagnosis of clinical endometritis, in 5 animals with confirmed VRE strains, the antibiotic susceptibility profile was performed to establish an appropriate therapy.

Enterococcus faecalis and *Enterococcus faecium* isolates were examined for comparative susceptibility to 9 antimicrobial drugs: amoxicillin, colistin, cefquinome, enrofloxacin, marbofloxacin, florfenicol, lincomycin, streptomycin, gentamicin, tetracycline.

The highest frequencies of resistance were for gentamicin, penicillin, streptomycin, tetracycline especially among *Enterococcus faecalis* isolates and for penicillin, gentamicin, tetracycline among *Enterococcus faecium* isolates (Table 2).

Table 2

Multidrug resistance of *Enterococcus spp.* isolated from dairy cow farms

	Number of isolates resistant to antibiotics									
Sample type	Total isolates tested	AX	CEQ	ENR	FFC	GM	MAR	P	S	TE
Enterococcus faecalis	2	2	2	0	1	2	1	2	2	2
Enterococcus faecium	3	1	0	0	0	2	1	3	1	3

AX-Amoxicillin, CEQ-Cefquinoma, ENR-Enrofloxacin, FFC-Florfenicol, GM-Gentamicin, MAR-Marbofloxacin, P-Penicillin, S-Streptomycin, TE-Tetracycline

Of the isolated VRE strains, more than 66.66% expressed resistance to three or more antimicrobials (gentamicin, penicillin, tetracycline, streptomycin, cefquinoma). Resistance to streptomycin and amoxicillin was observed in 100% of *Enterococcus faecalis* isolates. The lowest frequency of resistance to enrofloxacin, marbofloxacin and florfenicol was observed. All isolates showed resistance to at least one antimicrobial agent out of the 9 tested (Table 2).

The most commonly used basic drugs for the treatment of enterococcal infection are ampicillin, vancomycin (cell wall inhibitors) and aminoglycosides (gentamicin) (5). However, in the present study, resistance to these agents was observed: gentamicin (80%) and streptomycin (60%).

Enterococcus faecalis resistance to penicillins is most often caused by alteration of target proteins (especially PBP5) and extremely rarely by beta-lactamase production, which translates clinically in the absence of a benefit of combinations of penicillin with beta-lactamase inhibitors (ampicillin-sulbactam, amoxicillin-clavulanate) versus aminopenicillins (21).

Invasive infections caused by *Enterococcus faecium* have become increasingly difficult to treat in recent decades, as several classes of active antibiotics have gradually lost their effectiveness: first penicillins (especially ampicillin), then the emergence of high-level resistance to aminoglycosides, and to glycopeptides (13).

Resistance to penicillins and aminoglycosides may reduce the therapeutic spectrum available to clinicians for the treatment of enterococcal infections (17).

The fact that tetracycline is among the most used antimicrobial substances for the treatment of uterine infections in the two farms studied is also explained by the high percentage of resistance obtained after performing susceptibility tests.

Tetracycline resistance among enterococci isolated from humans and animals has been reported in several studies (1, 3, 6). The resistance of the isolates to gentamicin is also notable because it is an important representative of resistance to other aminoglycosides (2). These antibiotics and their analogues are frequently used because are cheap, affordable and commonly used in animal production sometimes without a prescription either for therapy, prophylaxis or in rations to promote growth (3, 6).

Isolation of enterococci in dairy cattle has the potential to reveal significant information about the role of animals as reservoirs of resistant strains (4, 5).

Enterococci can horizontally transfer genes of resistance to bacteria with implications in uterine diseases in dairy cows such as *Escherichia coli, Staphylococcus aureus* and *Listeria spp.* (25, 29).

In this research, the identification of *Enterococcus faecium* strains, a species frequently associated with nosocomial infection, illustrates its status of commensality, resistance and ability to survive and adapt to various stressors, such as antimicrobial use in the cattle breeding segment.

These isolated VREs represent a notable threat to public health because the same classes of antibiotics are used in the treatment of bacterial diseases in humans (19, 28).

Conclusions

In this research, most of the strains of the enterococci isolated were *Enterococcus faecium* followed by *Enterococcus faecalis*. *Enterococci* were mostly recovered by uterine samples of endometritic cows followed by pus and blood.

The identification of *Enterococcus faecium* and *Enterococcus faecalis* strains, species frequently associated with nosocomial infection, illustrates their status of commensality, resistance and ability to survive and adapt to various stressors, such as antimicrobial use in the cattle breeding segment.

One of the major risk factors in relation to colonization or/and infection with enterococci is antimicrobial treatment, especially given that very few antimicrobial agents can be used to control enterococcal infection due to its natural resistance to antibiotics and ability to acquire resistance from other bacteria.

Enterococci spp. among farm animals is a major health threat, as these resistant bacteria can be transmitted to humans through the via the food chain.

In conclusion, the fact that enterococci can cause drug-resistant nosocomial infections and are able to transfer virulence and genes encoding antibiotic resistance to others bacteria, suggest the need for continuous surveillance of *Enterococcus* strains in animals.

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REVIEW OF AVAILABLE SCIENTIFIC DATA REGARDING SEMEN PROCESSING AND ARTIFICIAL INSEMINATION IN DOGS

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Summary

Dog breeding and selection is increasingly important across the world. From beloved companions to police or military working dogs, detection, guiding or therapy dogs, there is an increased interest in obtaining better, healthier individuals, which preserve the important qualities and characteristics of the breed and are ideally free of genetically transmitted health conditions. In the effort of selecting dogs, the artificial insemination gained a great importance when planning the breeding as it offers the breeders the possibility to eliminate some limitations as distance, time spent travelling, synchronization of the travel with bitch fertile period, as well as to increase the gene pool in areas where importing dogs is not possible, or even to use semen from valuable individuals which are long dead. This study aims to review the current level of knowledge on semen processing and artificial insemination in dogs and to identify where current techniques need improvement. Artificial insemination with fresh and chilled semen usually leads to good results, but when comes to insemination with frozen semen the results are rather inconsistent in terms of rate of pregnancy and number of offspring. The oxidative stress during freezing process and cryopreservation has a direct, significant impact on semen parameters (viability, motility) and consequently on the results of artificial insemination. The available data suggest that Vitamin E and Vitamin B16 have an important antioxidant effect in case of refrigeration, but not in freezing and cryopreservation. Addition of kinetin seems to have beneficial effects in reduction of oxidative stress during the freezing procedure and cryopreservation. However, the control of oxidative stress during the freezing and cryopreservation remains an area to explore and improve. The proper correlation between the used semen type (fresh, chilled, frozen), artificial insemination technic and the timing for performing the procedure is essential for obtaining optimal results.

Keywords: artificial insemination, semen, dog, freezing, cryopreservation

The artificial insemination in dogs gained a significant importance in the last years as a result of the increased efforts of dog breeders in selecting better breeding stock for obtaining offspring free of health conditions or genetically transmitted diseases, with the required qualities for working as military, police, detection, guiding or therapy dogs and with typical breed qualities. Usually, the selection work exceeds the boundaries of one country, and very often the desired breeding dog is thousands of kilometers away, on a different continent. In other situations, it is used for breeding semen material of individuals which are long dead, but which are of a great value for maintaining the qualities of the breed. All these brought the semen processing and

artificial insemination in dogs in focus for breeders and for veterinarians across the world.

The current study aims to review the existing knowledge on semen processing and artificial insemination and to identify the areas where further research or improvement is needed. Unlike the processing and preservation of bull or boar semen, the dog semen is more susceptible to damage during processing (1). The quality of the semen that is going to be used for artificial insemination or processing is influenced by the status of the dog as such (age, health conditions, number of mattings or collections), but also by the collection technique and immediate manipulation of the semen (11, 16, 26). However, artificial insemination (AI) with fresh and even refrigerated semen leads usually to good results (19, 20, 26), but the real challenge is cryopreservation of dog semen and obtaining constantly good results in AI with frozen semen. The impact of oxidative stress on seminal material is important and generates lesions on sperm cell membrane, at acrosomal level and mitochondrial level (5, 7, 9, 22). All these lesions have impact on viability, motility and fertilizing capacity of sperm cells. Vitamin E and vitamin B16 that showed an important antioxidant and protective effect in refrigeration process, have no similar effect in freezing process (5). The use of glycerol in freezing is benefic but a correct balance between protective and toxic effect should be maintained (2, 4, 26, 28). The addition of egg yolk showed improved results in chilling and freezing process (7). However, there is still need for improving protection in order to obtain more consistent result. Different substances were studied for their protective effects (butylated hydroxytoluene, dimetilphormamide, clarified egg yolk), with various outcomes and lately the addition of kinetin showed improved protective effects in freezing procedure of dog semen (10, 12, 13, 23, 36).

For optimal results in terms of pregnancy and number of offspring, a proper correlation should be done between several factors: type of processed semen (fresh, chilled, frozen), type of artificial insemination (intra-vaginal, intra-uterine) and moment of bitch season (progesterone level, ovulation and maturation of the oocytes) (17, 26).

Quality of the semen – requirements and evaluation

The quality of the semen is of high importance for a successful semen processing in scope of preservation or for artificial insemination. Quality of semen is influenced by a number of factors: status of animal, collection conditions, manipulation of seminal material (11, 26). When comes to status of the animal, age and health condition of the stud are of great importance, but also the number of matings or sperm collections and the period between them. Ideally, the period between collections should be 2 to 5 days. A period longer than 10 days can lead to higher number of secondary abnormalities of sperm cells (11, 26) while a shorter period can lead to lower concentration of semen in ejaculate. However, for the males which naturally have a lower semen concentration, repeated collections in a limited period of time (daily or twice a day) are recommended in order to finally obtain a suitable concentration per unit of processed semen (straw or pellet). When a freezing

or shipping is planned for a male that was not collected or mated for a longer period of time, a prior collection should be done with 2 to 5 days in advance of the date of planned collection for freezing or shipping (11, 26). With respect to collection conditions, it is known that dogs can be easily collected. However, for a good semen quality, collection should be performed in appropriate conditions. The physical examination of the dog or any procedure that can stress the male should be performed after collection. Collection should be done in a separate room or isolated area, away of noise, without interruptions or presence of other individuals or animals. In most cases the presence of a bitch in season is not necessary. In order to stimulate the stud, a frozen-thawed swab with vaginal secretion of a female in estrus or, where available, chemical pheromone as methyl p-hydroxybenzoate can be used, by swabbing it on perineal area of an anestrus female teaser. However, the best results in quality of collected semen are obtained when the procedure is performed in the presence of a bitch in season (11, 26). Especially when scope of collection is semen preservation by freezing, the presence of a bitch in season allows obtaining ejaculates with higher concentration of sperm cells. As a method of collection, the digital manipulation is the most common one. Artificial vagina was used in the past, but it is not a current practice anymore. From the perspective of manipulation of collected seminal material, it seems that dog spermatozoa are quite resistant to chilling at 5°C but they are very sensitive to freezing (31). However, thermic shock should be avoided during manipulation of seminal material in the process of semen evaluation, preparation for chilling or freezing or artificial insemination (1). In this respect, according to some authors, all the materials that will enter in contact with seminal material should be kept at a constant temperature of 37°C (11, 26). Contamination of sperm with urine or blood should be avoided and evaluation and semen processing should be performed in the shortest time possible.

Semen evaluation should be done macroscopically and microscopically. Macroscopically, the volume, color, density and pH have to be assessed.

Volume of the entire ejaculate is mainly depending on the volume of third fraction (prostatic liquid). There are large variations based on breed, individuals, age, frequency of collection or collection method. The volume is usually measured using calibrated vials for semen collection. Decreased volume can appear in different prostatic or testicle pathologies (benign prostatic hyperplasia, prostatic cysts, inflammatory lesions) (11, 26).

Color of whole ejaculate is normally grayish-white. If only second fraction is collected, its color is white with a creamy appearance. Deviations from these colors indicate a contamination of the semen as a result of collection method or a pathological condition: green – grayish indicates the presence of pus, red-pink color indicates the presence of erythrocytes (coming from urethra, corpus cavernosum or prostate), yellow – contamination with urine, brown – presence of blood in the semen (11, 26).

Density is mainly evaluated when the fractionate collection is performed and is of interest for the sperm rich fraction (second fraction). The assessment of density

is quite subjective and this parameter can be affected by incomplete collection or contamination with prostatic liquid.

The semen pH should be assessed especially if the collected material is intended for refrigeration or freezing. A normal pH range for dog is between 6.8 – 7.4. In case that semen pH differs by pH pf the extender, the mixing of the two should be done very slowly in order to give the possibility to spermatozoa to accommodate with the modified pH (11).

Microscopic evaluation of the semen is usually based on motility, concentration, viability and morphology evaluation (11, 30).

Motility can be subjectively assessed, using a contrast-phase microscope with objective x20 - x40, a prewarmed slide on which a drop a semen is placed, covered by a coverslip, or more precisely, using the advanced technology for semen analysis (CASA system). However, due to the high costs of CASA system, in most veterinary clinics the subjective, classic assessment is performed. The progressively motile spermatozoa are the ones that are of interest (spermatozoa that cross the microscopical field) and their average percentage is calculated. A normal progressive motility in dogs is 70% (11, 26, 30). A lower value can be caused by the individual status of the male (too long periods between collections or systemic infections like Brucellosis) or by unproper semen manipulation (thermic shock, contamination with blood, urine or other substances). Agglutination of semen on the field always indicates systemic infections of the respective animal (26).

Concentration can be evaluated using a hemocytometer (Thoma, Thoma-Neu, Bürker, Bürker-Turke, Neubauer or Neubauer improved chambers). This cytometric method is considered the golden standard for concentration evaluation. The semen has to be diluted in order to reach a concentration that allows easy counting of sperm cells and, depending on the chamber used and the dilution applied a calculation formula must be applied. Some more advanced technologies can be used, such as spectrophotometer, flow cytometer or CASA system, but these are quite expensive. The normal sperm concentration in whole ejaculate in dogs usually exceeds $80x10^6$ spz/ml and in second fraction (collected separately or centrifugated) usually exceeds $200x10^6$ spz/ml, but there are many variations depending on breed, age, size of the testicles (11, 26, 30). A lower concentration in spermatozoa can be the result of some pathological conditions of the dog, but can also be the result of too many collections in a short interval or collection in the absence of a bitch in season as a teaser.

Viability (percentage of live – dead spermatozoa) is usually assessed by using an intra-vital stain (for example eosin-nigrosine stain). The stain will penetrate the impaired membrane of dead spermatozoa, staining them (11).

Morphology of the sperm cells is recommended to be assessed under phase-contrast microscope, but it can be also assessed under light microscope, on a stained smear (26). Different stains can be used, such as DiffQuik, Spermac, or eozine- nirgrozine. The examination should be performed under immersion objective

(x100 or x125). Minimum 200 spermatozoa should be counted in order to identify the abnormalities (primary or secondary abnormalities) (11, 30).

Diagnostic of infertility for a male should be decided after several collections and evaluations, but never based on a single collection (11, 26).

Semen processing

While the semen collected for the scope of immediate artificial insemination requires no processing and a minimal manipulation, the refrigeration in scope of shipment requires some processing and for freezing and preservation the procedure is quite complex and time consuming.

In case of immediate artificial insemination, the most important condition is not to expose the seminal material to thermic shock (11, 26). In this case, the prostatic liquid (third fraction) is beneficial, as it confers volume to the ejaculate and pushes the spermatozoa in vaginal and uterine tract. The whole ejaculate can be collected and used, or if fractionate collection is performed, the prostatic liquid should be used as extender for second fraction. In case of fresh semen insemination, deep vaginal AI is recommended, as the results in terms of pregnancy and number of offspring are comparable with the natural mating (25, 26).

Refrigeration of the semen is usually correlated with shipment of the material on medium distances of one -two days. The fractionate collection is required, as only the second, rich sperm fraction is of interest. In case that whole ejaculate is collected, it should be centrifuged for 10-15 minutes at slow speed (700G -1000G or 1000 – 1500 rotations/minute). The supernatant should be discharged and only the second sperm rich fraction will be used further for processing (18). Contamination of the second fraction with prostatic liquid should be avoided as the prostatic liquid has a toxic effect on spermatozoa at low temperature (6). In order to refrigerate the semen, a proper extender should be used. The extender should protect the sperm cells for cold shock, should provide the necessary nutrients (energy) and should maintain pH and osmolarity constant. Usually, extenders contain an antibiotic for preventing the bacterial growth. The most studied and used extender is Uppsala, a Tris-citric acid-egg-yolk-fructose based extender, that is prepared by veterinarians in clinic (21). There are also several commercial products (ready to use extenders) such as the ones from Minitube or CANINE- EXT.

In all extenders the addition of egg yolk in a proportion of 20% is needed. The egg that is going to be used should be fresh and from a controlled source, in order not to bring into extender any biological hazard. The egg yolk should be carefully separated by egg white and no egg white or egg yolk membrane should be mixed in the extender, as these have a harmful effect on sperm cells.

The mix of extender and egg yolk should be equilibrated from temperature perspective in order to have the same temperature as the semen. Some authors recommend 37°C as ideal temperature, others consider that room temperature is good enough. Next, the extender with 20% egg yolk will be slowly added in the sperm rich fraction and the whole content will be gentile mixed. The proportion between semen and extender should be 1:3 to 1:4 (5, 29).

The vial with the processed semen will be placed in a glass with water at 37°C and it will be placed in the fridge for slowly cooling to 4-5°C.

Depending on the type of the extender used, the refrigerated semen can be stored up to 10 days. Some studies reported longer periods with the condition to renew the extender after 10 days (35).

Freezing of dog sperm is a complex procedure that requires a significant amount of time. Still, there is an important demand for it across the world as this procedure allows unlimited preservation of seminal material (important in case of males with high genetic value) or shipment of semen at long distances.

As well as in case of refrigeration, only the second fraction of ejaculate will be used for freezing and it is extremely important to avoid contamination of this fraction with prostatic liquid (5, 14, 22, 32).

Dilution of the semen with appropriate extender, is a critical step for a successful freezing. There are several types of semen extender for freezing and the main difference between these and the ones used for refrigeration is the addition of a cryoprotectant agent such as glycerol. The semen can be extended in one or two steps, the last one being the preferred method, as it seems to be less stressful for spermatozoa. For each method dedicated extenders are available, such as CaniPlus Freeze 2 Steps from Minitube (3, 4, 34). However, the freezing process is accompanied by a significant oxidative stress which leads to irreversible lesions on spermatozoa, some of them visible at the moment of thawing, others not, but affecting in the end the fertilization capacity of the spermatozoa. Some tests were designed in order to predict the impact of invisible changes of spermatozoa, such as hypoosmotic swelling tests, with inconclusive results until now (15). In order to improve the protection against oxidative stress, different cryoprotective agents were investigated. The protective effect of glycerol is limited as in higher concentration has a toxic effect on spermatozoa (2, 12). Addition of Equex (detergent paste that improves the sperm cells survival after frozen-thawing) seems to allow higher glycerol concentrations in extender with good results in terms of semen quality after thawing (27). Butylated hydroxytoluene (BHT) added in semen extender at the level of 1.0mM improved the post-thawed sperm parameters (motility, viability integrity of plasma membrane and acrosomal integrity). However, higher concentration of BHT have negative effect (23, 36). Addition of egg yolk in semen extender have proved beneficial, while the addition of clarified egg yolk has no positive effect on sperm parameters (10). Addition of dymethylphormamide (DMF) was also studied; the concentration used in one of the studies (7%) had detrimental effects on integrity and function of plasma membrane, on acrosomal membrane integrity and mitochondria; a lower concentration (5%) showed similar protective effect with glycerol (2, 12).

A recent study evaluated the effect of kinetin supplementation (50 mM kinetin added in the extender) and the results suggest that kinetin has protective effect against oxidative stress, improving the motility, viability, and membrane integrity in thawed semen (28).

Freezing procedures are based on the freezing rate. In principle, the freezing can be done as fast freezing or slow freezing. Briefly, fast freezing consists of placing the cooled extended semen (usually packed in straws) horizontally, above the surface of liquid nitrogen; the distance between the semen and liquid nitrogen can vary from 3 to 5 cm and this variation will assure different freezing rates. The method is vastly used, as it is quite affordable. Slow freezing can only be done in a biological freezer, where straws are vertically placed, and the freezing rate can be fully controlled. The main limitation in using this method is the very high cost of biological freezer (7, 29).

The available data are not conclusive when comparing different freezing rates. On one hand, it is difficult to compare different studies as the conditions of the studies vary a lot (different extender used, different cooling rates, etc.). Moreover, some studies (33) showed that a slow freezing (with a decrease of temperature with 3°C/min from 4°C to -6°C, 10°C/min between -6 and -40°C and 50°C/min between -40 and -140°C) had negative effect on sperm motility compared with a faster freezing rate (temperature decrease with 3°C/min between 4 and -6°C and 50°C/min between -6 and -140°C). Another study (33) showed that total motility after thawing was significantly improved in slow freezing compared with fast freezing, but the straight-line velocity and spermatozoa average path were lower for slow freezing compared with fast freezing.

Correlation between type of semen used, AI technic and fertile period

Correlation between the type of semen used (fresh, chilled, frozen), artificial insemination technic and timing for performing the procedure, was investigated and there are consistent results showing that the proper correlation is of high importance.

The AI with fresh semen can be done either via deep vaginal deposition or trans- cervical deposition of semen (similar results) from day 1 to 4 post ovulation, in a range of progesterone level from 4 to 15 ng/mL. The expected intrauterine survival of spermatozoa in case of AI with fresh semen is 4 to 6 days (8, 26).

The AI with refrigerated semen can be done via trans-cervical deposition or deep vaginal deposition, in the days 2 to 4 post ovulation (ideally two inseminations performed) in a range of progesterone level of 8 – 15 ng/mL (8, 26). The expected survival rate for refrigerated spermatozoa in vitro is 24 to 72 hours.

In case of AI with frozen semen, the trans-cervical or intrauterine deposition is critical, as the expected survival period of spermatozoa is lower (12 to 24hours). Some studies indicate days 5 to 7 post ovulation, others day 3 to 4 post ovulation, but there is relative consensus on progesterone levels, which should be between 18 – 28 ng/mL (24, 26).

Conclusions

Semen processing and artificial insemination in dogs is increasingly important across the world. While the artificial insemination with fresh semen and even with refrigerated semen is performed with very good and mostly constant results, the cryopreservation of dog semen and subsequently the AI with frozen semen remains a challenge from the perspective of consistency of good results. The oxidative stress during freezing process has a significant negative impact on spermatozoa and their fertilizing capacity; further studies are needed in order to find appropriate protective solutions in terms of substances and proportions. The predictive potential of hypo osmotic test on viability and fertilizing capacity of spermatozoa should also be further investigated. The area of freezing procedures should also be further studied in order to identify the best freezing rate, in correlation with the extender used.

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EXPLORING THE CURRENT KNOWLEDGE CONCERNING THE BACTERIAL BIOFILM FORMATION AND ADHESION TO SURFACES

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Summary

Biofilms represent communities of microorganisms that start form the adhesion of planktonic cells to either inert or living wet surfaces, through a multifactorial process. The ability to form biofilms provides bacteria not only with vital nutrients, but also shelters them from harsh environmental conditions, antimicrobials and disinfectants. Microbial biofilms can be present in places ranging from the natural environment, to food-processing, industrial and medical settings. Pathogenic microorganisms also have the ability to form biofilms, and besides this, favorable environmental conditions in the food and medical sectors enhance the surface contamination by the pathogens. The presence of these bacterial communities in these sectors represents a major health safety issue, because by cross-contamination they can determine severe infections and treatment failures in humans, due to their antimicrobial resistance. Thus, in order to create efficient strategies against biofilms, we need to understand the bacterial adhesion and biofilm formation. Given the background, this review underlines the main mechanisms of bacterial adhesion and biofilm formation.

Keywords: biofilm, matrix, bacterial adhesion

In order to survive, the bacteria adapted and developed the ability to form biofilms. These microbial biofilms can be defined as homogeneous or heterogeneous bacteria communities living in a self-secreted matrix of extracellular polymer substances (EPS), that are present on both, inert and biotic wet surfaces (2, 8, 13). The biofilm shelters the microorganisms from xenobiotic stresses, it promotes the accumulation of vital nutrients, and enables the exchange of genetic material and signaling molecules (12, 13). Regarding the structure, EPS comprises around 90% of the biofilm, while the microbial cells represent at most 10% of the dry mass (12). In contract to the planktonic type, the bacteria from the biofilm are very resistant to heat, acidic or high salt concentration environments, antibiotics and other food preservatives (34).

Biofilms have been discovered on surfaces as various as rocks in rivers, roots, sea vents in the nature, water pipe systems and food-processing equipment in the industrial area, medical devices that are in the human body (i.e. catheters, orthopedic implants, heart valves) and on living tissues (2, 8, 22, 31).

Bacteria from the biofilms can be part of the normal microbiota of plants, animals and human, in which case they can sustain metabolic processes or even act against other pathogenic microorganisms (4, 18). In different industries, biofilms are considered useful and are being used for treating wastewater, bioremediation processes, producing biofuels, biofertilizers, and even additives for food and chemical applications (2, 33).

From another point of view, biofilms can be harmful to industries and human health, due to biofouling and corrosion, infections and diseases, respectively. Studies have reported that pathogenic bacteria in biofilms can both Gram-positive and Gram-negative, and single-species or multispecies. The formation of biofilm on inert surfaces in food-manufacturing environments represents a source of contamination for the products with *Salmonella enteritidis, S. aureus, Listeria monocytogenes, Bacillus cereus, Escherichia coli* serotype O157:H7, *Campylobacter jejuni* and *Cronobacter sakasakii* (5, 7, 31). Da Silva et al. pointed out biofilm-forming pathogens that are typically related to nosocomial infections, such as *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Candida albicans*. Besides these species, *Escherichia coli, Enterococcus faecalis, Staphylococcus epidermis, Mycobacterium tuberculosis, Klebsiella pneumoniae, Bacillus subtilis, Neisseria gonorrheae, Streptococcus viridans, Helicobacter pylori, Proteus vulgaris and Proteus mulbaris* were also listed as pathogens associated with life-threatening infections in humans (8, 22).

Considering all aspects, we can understand the importance of managing microbial biofilms both to improve the industrial applications by supporting their formation and to prevent harmful actions by disturbing their development (2). In order to do this, it is vital to have a better knowledge of the steps and mechanisms that are involved in biofilm formation.

Therefore, in this review we emphasize some of the most compatible studies that will grant us with better insight regarding biofilm formation and the factors that affect adhesion and initial attachment of the bacterial cells to surfaces.

Biofilm formation

The formation process of the biofilm is complex and requires a certain type of signaling, also known as quorum sensing (QS), continued with the transcription of different sets of genes that differ from the planktonic genes of the same species of bacteria (20). Biofilm formation is triggered by the environmental conditions, resulting with the bacteria adhering to a surface, biotic or abiotic (16). The triggering conditions vary among the bacteria species and serotypes. Thus, some bacteria can form biofilm in many situations, while other required more particular conditions. For instance, some strains of *Escherichia coli* will form biofilm in limited-nutrient conditions, while *P. aeruginosa* in any situation (24).

The biofilm formation mechanism is complex, with some particularities among microorganisms. In various studies, the number of steps it takes for the biofilm to form is a subjective topic, ranging from four to five steps. Therefore, it is up to each author to choose the structuring that pleases them the most, regardless of the fact that the general steps are similar, and as follows: (I) adherence of planktonic cells to the surface, (II) formation of the microcolony, (III) biofilm maturation, (IV) dispersion, resulting in colonization of other surfaces (8, 20).

1. The initial adhesion

In order to colonize, the bacteria must transition from the planktonic form in the bulk liquid, to a immobile state, attached to the surface. The first step towards this transition is to reach the surface and initiate adherence by a few cells (2). Planktonic bacteria in liquid environments can advance towards a surface either actively, by their own movements, or passively, being subjected to physical forces (3). Undoubtedly, the most efficient is the active motility, which is realized by the flagellated bacteria. They can swim directly to a surface as response to environmental stimuli, such as light, oxygen, temperature, chemical signaling and magnetic fields (30). In example, via chemotaxis *Vibrio* spp. can adhere to chitin, a major component of the arthropods, crustaceans and molluscs, and also metabolize it (23). In another study, *Bacillus subtilis* cells were shown to be attracted by other bacteria, within a surface-associated biofilm, via electrical signals. The latter one is done by releasing potassium ions from the biofilm, which regulates the motility of cells towards the microbial community by modifying their membrane potential (19). The initial attachment of the bacteria is also affected by the high flow velocity in the bulk liquid. The swimming behavior of *E. coli* over a surface was affected by a high flow and shear, whereas the bacteria managed to advance towards the flow under a moderate shear rate. This could be an explanation to the propagation of *E. coli* cells in catheters, rivers or water pipes (21). The near surface swimming of *Vibrio cholerae* cells was reported in a study to be affected by mannose-sensitive haemagglutinin pili, which realize intermittent contacts with the surface, therefore increasing the time spent near it (32).

The adherence and passive movement of the bacteria towards the surface is regulated by attractive and repulsive forces, hence if the latter forces are exceeded by the attractive ones, the initial attachment between the cell and the surface will succeed (28). In the following we will highlight the forces that bacteria will encounter while transitioning from the liquid to solid environment, as follows: van der Waals interactions, which act at the longest ranges and are generally attractive; hydrophobic interactions, which, depending on the chemistry structures of cell, surface and medium, can be either attractive or repulsive; electrostatic interactions, which depend on the pH and ionic strength of the liquid medium; and steric forces (28). Regarding the latter of the forces, bacteria, such as *E. coli*, *Pseudomonas*

aeruginosa and *Pseudomonas putida*, are able to generate long-ranging steric forces through their surface polymeric brush layer, thus enhancing their adhesion to surfaces (1, 6). Hydrophobicity and charge also greatly influence the interaction with surfaces, as the bacterial cell envelope is often negatively charged, positive to neutral charged surfaces are more likely to be colonized, in opposition to the negatively charged. In addition to this, as another general rule, hydrophobic bacterial cells will prefer hydrophobic substrates (2).

Apart from the physicochemical properties of the surface, there are other factors that influence the efficiency of adhesion. To begin with, in liquid medium, molecules that come from both the environment and as a result of bacterial metabolism, will adsorb on the surface and create conditioning films, which may increase surface adhesion by attaching to the binding sites on the bacterial cell (15). As an example, *P. aeruginosa* specimens place a trace of Psl exopolysaccharide, when contacting a clean surface, to which other cells will attach, creating starting points for microcolonies (34). Moreover, topography of the surface is also important to the adhesion mainly because it can alter the hydrophobic characteristics and that any microscopic irregularities of the surface will support bacterial adhesion (28).

Initially, the attachment is reversible, due to weak connections between the bacteria and surface. If the repulsive forces are greater than the attracting ones, or the nutritional substrate is not available, bacteria can detach and return being planktonic. The remaining microorganisms will start preparing for a long-time attachment, through secreting adhesins and adhesive appendages, thus realizing the irreversible attachment (16).

There is a variety of pathogens that overcome repulsive and hydrodynamic forces with the help of the flagella, the importance of which being well documented for *P. aeruginosa, Listeria monocytogenes, E. coli* and *Vibrio cholerae* (23, 24). Besides propelling the bacteria to the surface, the flagella also acts as an adhesin by realizing physical contact with the substrates; also through it the microorganisms can reach places inaccessible to them, due to cell size. The initial adhesion of flagellated *E. coli* on microscopically rough surfaces can be explained by their extra accessibility into crevices (14).

The adherence on different surfaces is due to type IV-pili in *P. aeruginosa* species (20), type I pili in *E. coli*, and type IVc tight adherence pili in both *Caulobacter crescentus* and *Agrobacterium tumefaciens* (26, 27).

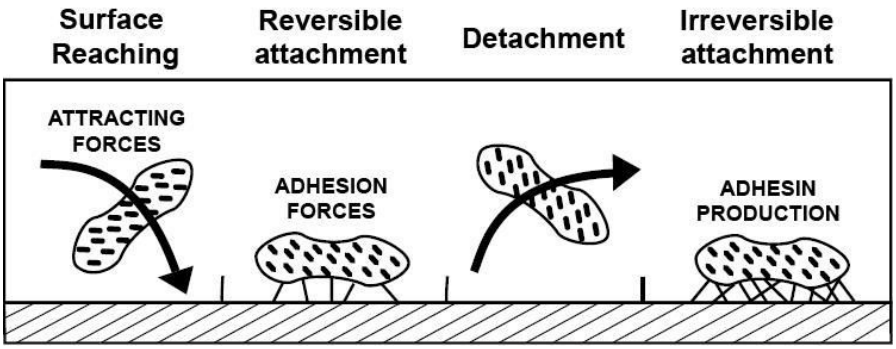

Fig. 1. Movement of the bacteria towards the surface, initial attachment, desorption, and irreversible attachment (11)

2. Microcolony formation

The adhesion to the surface will trigger responses which lead to the transcription of a different set of genes, compared to the planktonic genes, therefore enhancing the bacteria's expression of adherence factors (23, 25). Many microorganisms have the capacity of producing and secreting slimy substances necessary for the biofilm matrix, this ability being activated by specific conditions, depending on the bacteria species (11). Therefore, cell adsorbed on surfaces will secrete QS molecules, EPS, and secondary metabolites that dictate cell motility, growth, pathogenicity and biofilm formation (29).

Autoinducer molecules are signaling molecules, participating in cell-to-cell communication, regulating the formation and then dispersion of biofilms. N-acyl homoserine lactone is produced by Gram-negative bacteria to communicate, in contrast to the Gram-positive bacteria that use modified peptides. The two large groups signal each other through the autoinducer-2 molecule, which is synthetized by the enzyme LuxS (9, 17).

During this phase, the microorganisms produce a small quantity of the biofilm matrix, begin multiplying and forming small cell groups known as microcolonies (23, 25).

The microcolony formation in two mutant classes of *P. aeruginosa* was studied. One of them was mutant of motility and flagella, meaning that it could not adhere to surfaces and the second one was mutant of type IV pili. In the end, the mutant of type IV pili was unable to establish microcolonies, thus pointing out the importance of this cell component in microcolony formation (16).

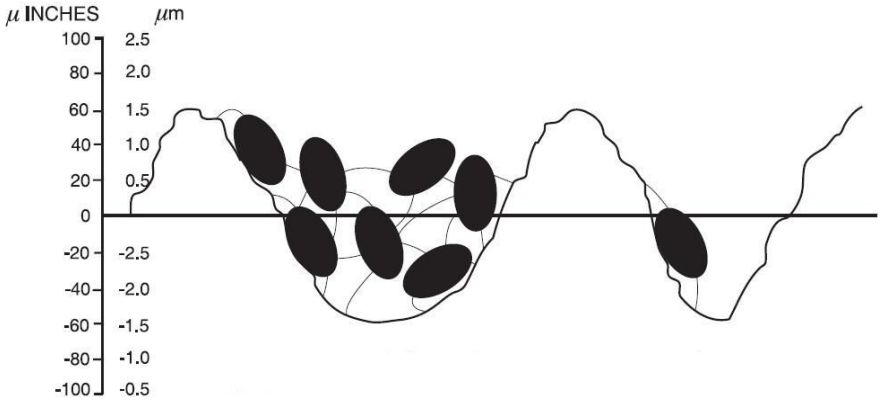

Fig. 2. The adhesion and microcolony formation on rough surfaces (11)

3. Biofilm maturation

After the microcolony was realized, bacteria cells will start signaling each other through autoinducer molecules, which will activate the genes required for synthetizing extracellular polymeric substances (EPS), necessary to the biofilm matrix formation (22). In *P. aeruginosa*, the stability of biofilm is provided by alginate, pel, and psl, which are polysaccharides (23), while in *Vibrio cholerae* the organization of biofilm formation is regulated by polysaccharides and three proteins (RbmA, RbmC, and Bap 1) (8).

Furthermore, studies have discovered that extracellular DNA (eDNA) is involved in stabilizing the biofilm matrix by establishing cell to cell connection. The biofilm maturation hereby consists in two phases; the first phase comprises the cell to cell communications with the result of autoinducer molecules production, and the second phase represents the activation of all genes required for the EPS matrix, and then transformation of microcolonies intro macrocolonies (22).

The mature biofilm is like a living tissue, a complex cooperating community, metabolically active, comprising of multiple species of bacteria, each armed with different sets of enzymes and metabolizing nutrients that no species could digest alone. Approximately 75-95% of the biofilm is composed EPS matrix, the rest of it being represented by microorganisms and different substances. Biofilms can be arranged on two level as follows: a superior level (aerobic level), and a bottom level (anaerobic level) (11).

Dispersion

In order to colonize new areas, bacteria from the biofilm will get into a mobile state via dispersal from the matrix, resulting in a new start of biofilm formation in a different area (16). Biofilm dispersion can be both, passive, or active. In the first instance, it is the result of shear stresses and aggregates simply slough off, while in the case of active dispersion, bacteria can decide whether to continue living in the present microbial community or to start a new biofilm formation (23). It is worth to be mentioned that during passive dispersion, there is a high possibility that bacteria aggregates retain the biofilm characteristics, such as antimicrobial resistance, whereas the bacteria that are actively dispersing might return to the original planktonic phenotype (10).

There are different substances that are reported to induce biofilm dispersion. In the case of *P. aeruginosa* the enzyme alginate lyase and the increasing amount of carbon and nitrogen sources facilitate dispersion. Moreover, the increased levels of c-di-GMP molecules are indicating microcolony formation, while the decreased levels are correlated with enhanced bacterial motility (23).

Conclusions

In the present review, we have outlined the factors that control the bacteria initial attachment and adhesion to surfaces, followed by the biofilm formation. Fortunately, the collaborations between scientists resulted in new ways to study the early stages of bacterial biofilm formation, such as microfluidic devices, atomic force microscopy, microscopy and molecular biology techniques combined with differential staining with viability dyes.

After analyzing all of the chosen relevant studies, we could agree to the fact that is difficult to draw a main conclusion regarding how the bacterial attachment is achieved in a common way, for the reason that all participating elements (liquid environment, bacterial cell, and surface) are ever changing. In addition to this, depending on every bacteria species, there are different extracellular structures that participate in the adhesion process, either favoring, or hindering it. Therefore, a slight modification to one of the elements can greatly impact the way in which factors interact with each other.

Despite the difficulty, we can draw conclusions regarding the fact that, generally, bacteria are inclined to colonizing surfaces with certain characteristics, such as hydrophobicity, presence of microscopic irregularities, and more importantly, an existing conditioning film, but this behavior does not apply to all bacteria.

In a short time after adhering to the surface, the bacteria will multiply and create, through signaling and physiological changes, multicellular communities within an extracellular matrix secreted by them. These communities will continue

growing and become complex biofilms. The population within the biofilms is dynamic, due to bacterial cells joining or dispersing constantly.

Understanding the complex mechanism of biofilm formation is essential for preventing its presence in areas, such as food processing units of medical devices, and thus, reducing the health risks related to biofilm-forming pathogens. The new technology and techniques of investigating cell attachment and biofilm formation will contribute to a more detailed perspective of how different factors control the bacterial biofilm formation and adhesion to different surfaces.

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A RARE CASE OF CORNU CUTANEUM ON THE SKIN OF A CAT'S NECK

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Summary

Cornu cutaneum is a clinical term that describes unusual keratinous skin tumors with the appearance of horns extending vertically from the skin. At dogs and cats it is less found and usually occurred on the scalp, paws and pads. In our paper we presented an unusual finding of cornu cutaneum on the skin of a cat's neck. Therapy was surgical excision of the growth was performed under general anesthesia (ketamine / xylazine). A broad-spectrum antibiotic (shotapen) was given, and the wound healed without complications per primam.

Keywords: cornu cutaneum, cats

Cornu cutaneum is a clinical term that describes unusual keratinous skin tumors with the appearance of horns extending vertically from the skin (1, 11, 14). They projecting from the surface of the skin, being movable in the skin and often hang downwards. They are usually small and localized but can, in very rare cases, be much larger. Although often benign, they can also be malignant or premalignant (3, 4). The cause of cornu cutaneum is still unknown, but it is believed that exposure to radiation, some viruses and etc. can trigger the condition. Cornu cutaneum are relatively frequent in cattle, sheep, goat and birds, less frequent in horses and rare in pigs (5, 6, 7, 13). At these animals usually predilection site is being head, near to medial canthus of eye, ear, udder scrotal region and limb (2, 14, 15). At dogs and cats it is less found and usually occurred on the scalp, paws and pads. In our paper we presented an unusual finding of cornu cutaneum on the skin of a cat's neck.

Materials and methods

History: A cat named Deminutiv, aged 2.5 years, was brought to a clinical examination in an outpatient clinic due to a growth on the skin of the neck.

Clinical finding: the cat is afebrile t = 38.6^{o}C, uniform breathing, normal mucosa color, preserved skin turgor, cheerful with normal performance of physiological functions. On the dorsal side of the neck, a horny growth of the skin looks and the strength of the nail is about 1 cm in size. No changes were noticed on other parts of the body.

According to the owner, the growth started to appear some 6 months ago, and on the recommendation of the local veterinarian, she applied antibiotic ointment to the changed place twice a day. The change has grown to its current size and is

not painful on palpation. Grossly, the growth was grayish-black in color, firm and hard to touch and projected above skin like a horn. The stalk of the so-called horn was covered with soft thick skin, which was continuous on one side, scurfy on the other sand hard at the base on neck (Fig. 1A, B).

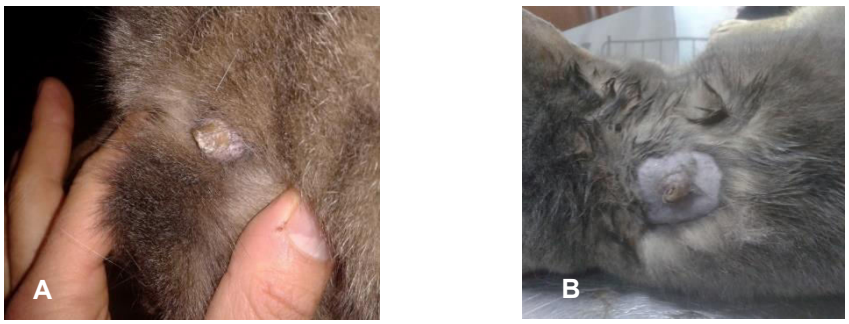

Fig. 1. Cornu cutaneum base on neck of cat

Diagnosis: cornu cutaneum in regio cervicalis in obs.

Therapy: surgical excision of the growth was performed under general anesthesia (ketamine / xylazine). A broad-spectrum antibiotic (shotapen) was given, and the wound healed without complications per primam. After 10 days, the sutures were removed.

After extraction of cornu cutaneum (Fig. 2 A, B, C) tissue sample was preserved in 10% buffered formalin for histopathological studies.

Fig. 2. Extracted cornu cutaneum

The paraffin embedded tissues were cut into 4-5 micron thick sections and stained with hematoxylin and eosin. On the cross-section is observed extensive, compact and laminated hyperkeratosis with numerous keratinocytes and acanthosis (Fig. 3A, B).

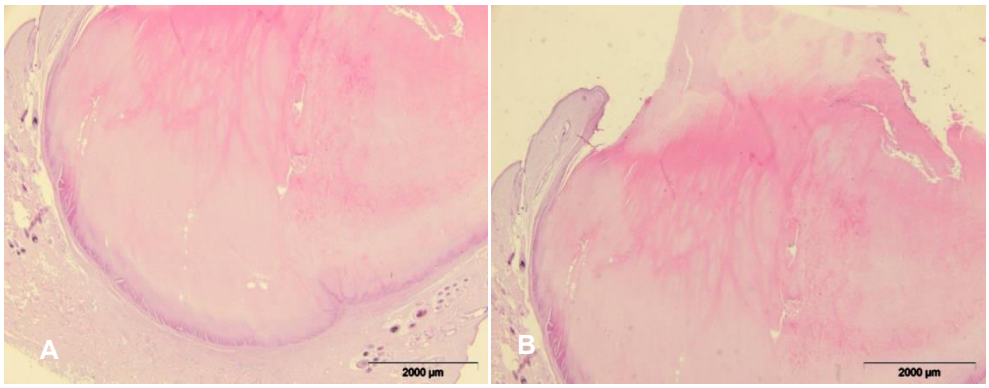

Fig. 3. Extensive, compact and laminated hyperkeratosis with numerous keratinocytes

Results and discussions

Cutaneous horns have been associated with feline leukemia virus (FeLV) infection, sarcoma feline (16), papillomas, trichoblastomas, squamous cell carcinomas, follicular cysts, keratinizing acanthoma infundibular or actinic keratoses (9,12,14); however, in some cases, such diseases are not identified (11). There is not reports in the literature of the association of cutaneous horns to the CRVF. In this report, there was no evidence of infection by feline leukemia virus or neoplasia. Cutaneous horns are not frequent in cats, mainly in young cats (1, 9). In that case, it was going to suggest a congenital origin (11, 16).

Histologically there is hyperkeratosis extensive and compact blade, but the base of the lesions is characterized by parakeratosis, apoptosis and cells multinucleated keratinocytic giants. Injuries below the nails can be seen in FeLV negative cats and have different histological characteristics (4, 8, 11, 17). Histopathological evaluation revealed a benign disorder, corroborating the study de Copcu et al. (3), who stated that the cutaneous horns are predominantly benign. Although the cutaneous horns can occur in several places, it is important to remember that in cats, these have a regular location on the cushions, and may occur on palm and plantar cushions (9). Occurrence of cornu cutaneum on the skin of a cat's neck and present an unusual finding location.

In view of the rarity of reports of this disease, this study contributes through the description of a case of cutaneous horn of origin unknown in a feline, with a suggested origin congenital. No lesion was found malignant skin or association with infection by FIV, thus representing a favorable prognosis for the patient.

Conclusions

Cornu cutaneum is a clinical term that describes unusual keratinous skin tumors with the appearance of horns extending vertically from the skin. At dogs and cats it is less found and usually occurred on the scalp, paws and pads. In described case presence of cornu cutaneum of the skin of a cat's neck was unusual finding since it does not represent a typical place where it is located.

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