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VETERINARY MEDICAL USE OF CANNABIS, CANNABINOID RECEPTORS AND ENDOCANNABINOIDS SYSTEM IN MAMMALS

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Summary

The use of medical cannabis was proved since ancient when the emperor Shen Nung wrote a book in 2737 BC about medical benefits of cannabis. Historical evidences from India, Greek and Egypt explained and presented the effectiveness of cannabis use in different medical conditions: cancer, non-specific chronic pain, multiple sclerosis, epilepsy, gastrointestinal disorders, drug addiction, post-traumatic stress disorders, inflammation, acute pancreatitis, and oxidative stress. In medicine, Cannabis plants are not recognized or regulate approved as medicinal plant. The main components of cannabis (*Cannabis sativa*) which are important from medicinal point of view are tetrahydrocannabinol (THC) and cannabidiol (CBD) because both components interact with cannabinoid and other neurotransmitter receptors found mainly in brain. The active form of cannabis used in medicine is CBD, while THC causes intoxication and euphoria. In U.S.A., Food and Drug Administration approved in June 2018 one drug (Epidiolex) containing CBD as active ingredient for treatment of severe epilepsy forms in humans. If in humans the cannabis can be used in medicinal purpose, why cannot be promoted as treatment in animals with severe medical conditions? Literature data present the use of CBD oil, tincture, soft chews or tablets in dogs, cats and horses, which are legal in several states and is also safe. Known as cannabinoids receptors (noted as CB), CB1 and CB2 are lipophilic G protein-coupled receptors, recognized as cell membrane receptors, being part of endocannabinoid system. The cannabinoids receptors can be activated by endocannabinoids from mammals, by cannabinoids from plant (*Cannabis sativa*), or by synthetic cannabinoids. Prescription of CBD products for animals has to take in consideration if the products are organic or not, the concentration in CBD, and if the products are free of THC.

Keywords: cannabis, veterinary medical use, CBD, THC

Since ancient, in 1898 Dr. Stockwell G. Archie noted the use of cannabis as a primary treatment for diseases like chronic pain, epilepsy, brain tumours, asthma, eczema, anorexia and even to "promote mental cheerfulness". He discovered some of the therapeutic properties such as: anti-dolorific antispasmodic, nerve stimulant, and also can be used as diuretic, aphrodisiac and oxytocic (7, 30).

Knowledge of cannabinoids' biochemistry and pharmacology evolved over an extensive period, until the last two decades, when marijuana research was a rather "esoteric field of interest to a small number of scientists", but it has accelerated dramatically in recent years.

The Cannabis is the dried inflorescence of female plants of *Cannabis sativa*. The Cannabidiol (CBD) is the component used in many current medical fields, which is legal in many countries, even some European countries. But, every variety contains different psychoactive substances, drugs or not, including the most important: the Δ^9 -tetrahydrocannabinol, commonly known as Δ^9 -THC or THC (11, 34). This component makes the plant illegal in many countries due to its concentration of THC, which can be harmful if it is used for a long-term or it is used in other than medical purposes (18, 28). There are different varieties of cannabis which are legally cultivable in some countries, with very good specific regulations (5).

Cannabis plant

The cannabis plant is an adaptive and hardy annual hemp plant, which grows in many temperate and tropical zones of the world. It can reach a height up to five meters during a four-to-six-month growing season and is dioeciously, occurring as male and female. A typical female cannabis plant produces hundreds of very small flowers which are clustered in a large mass at the top of the plant which can reach a meter height. The highest concentrations of the psychoactive component in cannabis is contained in the buds (the flowering tops of the female plant), followed by the leaves. Stalks and seeds have much lower concentrations of psychoactive components compared to buds or leaves. Flowers in the cannabis plant contain a single curled leaf, covered by large numbers of hair-like gland-cells called trichomes. When the trichomes are ruptured, resinous oil is released. This oil contains high quantities of active compounds, including the psychoactive components of cannabis (3, 13).

Chemistry of cannabis

Cannabis contains different chemical substances, most belonging to cannabinoids class. The cannabinoids are terpenoids – non polar molecules with low solubility in water. The concentration of psychoactive cannabinoids is around 1-10% in the plant and it can reach 60% in resin and oils.

Cannabigerol (CBG) is the cannabinoid precursor of tetrahydro-cannabinol (THC) and cannabidiol (CBD). CBG appears to react with receptors, other than those from the endocannabinoid system (36). It has been suggested that it is benefic for treatment of cancer, inflammatory bowel disease and has good antiseptic properties. However, research on its therapeutic efficacy is at an early stage.

Cannabinol (CBN) is a by-product of the oxidation of THC, non-psychoactive, being considered less-potent cannabinoid (14).

Cannabis regulations in different countries

In different countries there are three kind of legalization: for medical use, recreational use or research use. Cannabis has been used for therapeutic

purposes, but from the 19th-20th centuries started to be used in medicine, coinciding with international efforts to prohibit its recreational use. The Fatality Analysis Reporting System gave data regarding the effect of using marijuana in U.S. drivers, which showed a high percent of fatal crash in the last 20 years (32). However, since the late 1990's, a number of states and countries have moved back prohibitions of cannabis for patients suffering with serious medical conditions (25). A number of international jurisdictions allow now the cannabis use for medicinal purposes, including Canada, Czech Republic, Finland, Germany, Israel, Italy, The Netherlands, 23 states of the United States of America and a further 12 U.S. states allowed the use of low THC, high-CBD cannabis, in some cases for research only (6, 38). In addition, Uruguay and the U.S. states of Alaska, Colorado, Oregon and Washington have legalized cannabis for recreational use. On June 25, 2018, Food and Drug Administration (FDA) from United States approved the first drug – Epidiolex, containing an active ingredient derived from marijuana (cannabis) to treat rare, severe forms of epilepsy. The Epidiolex is a drug containing cannabidiol (CBD), as an oral solution recommended for seizures associated to rare and severe forms of epilepsy, Dravet syndrome, Lennox-Gastaut syndrome (40).

Romania regulation did not legalize marijuana for medical use, but allowed marijuana derivatives for medicinal purposes only. It is illegal for recreational use and is allowed in only limited form for medicinal purposes. Tests are currently underway to determine the efficiency of marijuana administration for patients suffering from Alzheimer's disease, Parkinson's disease, Huntington's disease, and Tourette syndrome (23).

In Italy the use of cannabis is illegal; the exclusively personal use is decriminalized but punished with administrative sanctions, such as: suspension/revocation of the license, the firearms license, the residence permit and others (39). The medical use of cannabis is legal under prescription, as well as the cultivation of Cannabis sativa for strictly textile use and with very low content of Δ^9 -tetrahydrocannabinol (THC) in flowers to obtain hemp fiber and occasionally also food products or cigarettes. All without significant presence of the psychoactive principle THC and therefore of free sale; usually these products contain as a main ingredient cannabidiol, a legal substance as not psychoactive but only with sedative effects. The legality limit for the sale of such derivatives and unworked cannabis parts is a maximum of 0.6% THC (4).

Medical use and pharmacological properties of cannabis

Cannabis contains different chemical compounds, some of which are classified as cannabinoid, and some of which are specific to the plant. There are more than 100 such compounds and some 300 non-cannabinoid chemicals in the cannabis plant (11, 30). In addition, there are compounds about which less is known, such as terpenes and flavonoids (its flavour and fragrance components), which are thought to have a broad spectrum of action, including anti-oxidant, anxiolytic, anti-inflammatory, anti-bacterial, anti-neoplastic, and anti-malarial (20).

However, there are several species within the Cannabis genus, and within those species a number of different strains which contain varying amounts of the different cannabinoids and therefore can be used to treat different medical conditions. Some strains contain extremely low amounts of THC cannabinoid and are used to treat epilepsy and therefore are not considered to be psychoactive. Cannabis in medicinal use can be administered in various ways, but oral or inhalation or smoke are the most commonly (34).

The alternative to treatment with the raw product or its less refined extracts is the most suitable of refined cannabis-based medications produced by pharmaceutical companies. Various forms of cannabinoid medications have been approved by government authorities in different parts of the world. The advantage of such products is that their constituents are known and their dosages can be titrated with accuracy by prescribing medical practitioners. They are most commonly administered orally or by oromucosal spray (19). The disadvantage of such products is that impediments persist in relation to their accessibility by reason of regulatory structures and their high cost. Pharmaceutical natural cannabis extracts can be distinguished from synthetic cannabinoids, because are extracted from cannabis plant, not synthesized in laboratory from other chemicals.

Endocannabinoid system

The endocannabinoid system (SEC) is composed by CB1 (primarily in central nervous system) and CB2 (peripheral tissues) receptors, which are activated by the endocannabinoids (Anandamide and 2AG), secreted by fatty acids (11, 24).

Raphael Mechoulam has been one of the first researchers who isolated and synthesized the cannabinoids from the plant, and studied experimentally the THC and then CBD (15). The THC and CBD can enter into the organism and can stay activated for a long time.

The Δ 9-tetrahydrocannabinol (THC) has the capacity to activate CB1 and CB2 receptors, having the capacity to activate the entire endocannabinoid system. It is found in the trichome that covers the dried flowering tops and leaves of the female plant. THC is high lipid soluble and in animals and humans is distributed in different organs, such as: liver, brain, and kidney, but is present also in fat tissue. Biliary excretion is the highest THC excretion way, and also about 15% of THC is excreted through the urine (11, 20).

The cannabidiol (CBD) does not have the capacity to activate the receptors (CB1-CB2) but it is essential for the inhibition of the FAAH enzyme (fatty acid amide hydrolase), avoiding the destruction of anandamide (21). CBD eliminates or mitigates some of the effects of THC, moderating its psychoactivity and reducing the incidence of THC-induced sedation, anxiety and tachycardia.

Endocannabinoid system works on humor, sleep, appetite, metabolism, pain, memory, inflammation, digestion, reproduction, neuroprotective functions,

immunitary functions. CB1 receptor – primarily in central nervous system, is more concentrated in the nervous system. It works on pain, appetite, and emotive process. CB2 receptor – in peripheral tissues, is more concentrated in immunological cells, gastrointestinal level, and peripheral nervous system. It works most on all inflammation processes.

Experimental studies of cannabinoids in cell cultures and living organisms

Autophagy can lead to cell survival or cell death. Regarding to this, it has been demonstrated that $\Delta 9$ -tetrahydrocannabinol induces human glioma cell death due to autophagy stimulation. Experimental data indicate that THC induced ceramide accumulation and also eukaryotic translation initiation factor 2 α phosphorylation and thus activated a stress response that promoted autophagy. In vivo experiments demonstrated that in cannabinoid-induced human and mouse cancer cell death autophagy is upstream of apoptosis. Activation of apoptosis is necessary for cannabinoids antitumor action. These findings describe a THC mechanism which can promote the autophagy of cancer cells and provide strong evidences that cannabinoid administration may be a good and effective therapeutic strategy for targeting human cancers (31).

Slavic and his collaborators observed in their researches that rimonabant – which blocks the CB1 receptors – had cardioprotective action in rats with metabolic syndrome (33). Di Filippo and his team demonstrated that administration of one synthetic CB1 and CB2 receptor agonist significantly decreased the infarct size in mouse model with myocardial ischaemia (9, 17). Also, Batkai in 2004 reported that in rats with hypertension the endocannabinoids suppressed cardiac contractility (2). In 2007 an experimental team coordinated by Mukhopadhyay observed that treatment with rimonabant improved cardiac dysfunction and significantly protected against doxorubicin-induced cardiotoxicity in mice (26).

Experiments using cannabinoids demonstrated very good effect on pain management. Thus, long-term treatment with a palmitoylethanolamide – which is an endogenous cannabinoid-like substance in the central nervous system, significantly reduced allodynia and hyperalgesia (22). Other study demonstrated that administration of cannabidiol in rodents suppressed the chronic inflammatory and neuropathic pain (37).

The THC and CBD exhibited good potential therapeutic effect in the allergy (asthma) treatment by inhibiting the critical T cell cytokines expression (12, 16).

Other experimental researches proved beneficial effect of THC administration in animals vomiting due to the cisplatin or lithium treatment (8, 27).

Food intake, body weight management and diabetes were also experimentally studied in relation with cannabinoids administration. Also, El-Remessy demonstrated that cannabidiol reduced inflammation, neurotoxicity, and blood-retinal barrier breakdown in diabetic rats (10), while the administration of

CBD in young non-obese mice significantly decreases the diabetes incidence (35). CB1 receptor is a key regulator in lipids metabolism, in immunologic system and also in sympathetic nervous system. Endocannabinoid system has a very important role in energy balance involving different mechanisms. When the CB1 receptor is activated, the adipogenesis and lipogenesis are activated, altering the mitochondrial function in diet-induced obesity. There are some compounds used as therapeutic CB1 antagonists (as rimonabant) that increase the energy expenditure, activating the fatty acid oxidation and lipolysis, leading to severe body weight loss. But this is associated with various side effects such as anxiety and depression, primary due to the action of the therapeutic compound to the brain (29).

Some studies, but not a large number of scientifically published from this field, described the utility of using medical cannabis in associated rheumatology treatment, but the greatest problems in this medical approach are the doses, frequency and administration method (1).

There are studies also on animal epilepsy and seizure treatments, especially on dogs and cats. In animal treatments the most often administration is as cannabis oil or spray based on cannabis extracts.

Conclusions

Cannabinoids are chemical components present in cannabis plant, with different variations depending on the botanical part of the plant, the flower and leaves being the most concentrated in psychoactive components, especially in THC.

Medical cannabis, more exactly the cannabidiol (CBD), is used in various treatments with very good results since ancient. During the last decades researchers tested the effect of medical cannabis on different cell cultures and on different experimental animals. Also, the cannabinoids were used in human treatments of different disorders, and the results demonstrated the efficiency, this becoming an alternative medical approach.

Mostly the cannabinol is little studied, being the metabolite of Δ^9 -tetrahydrocannabinol (THC), acting as antagonist of CB1 receptors, which alters the energetic and lipid metabolism, influencing also the nervous system activity.

The regulations of medical use of cannabis in different countries are different due to the content of cannabis in cannabidiol (CBD), cannabinol (CBN), Δ^9 -tetrahydrocannabinol (THC), cannabigerol (CBG) and others; and also the legislation involve doses administration, frequency administration, and very important the administration type (extracts, oil, powder, transdermal patches, etc.).

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EPIDEMIOLOGICAL ASPECTS OF SPLENIC TUMORS IN DOGS: A RETROSPECTIVE STUDY

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Summary

Splenic tumoral pathology in dogs has a high prevalence and is often diagnosed late. For the present study we reviewed records of canine patients submitted to the Pathology department between April 2007 and June 2018. Gross inspection and histological analyses were performed. According to our results, splenic tumors represented 6% (97 cases) of the total tumors diagnosed in canine patients (1629 cases). Splenic tumoral lesions were histologically diagnosed as malignant (89%) and benign tumors (11%), hemangiosarcoma being the main malignant tumor diagnosed (75%), followed by splenic lymphoma (22%). Of the 97 splenic tumours included in the present study, 64 were diagnosed as primary splenic hemangiosarcoma and 56% had metastases in different organs. Regarding breed predisposition, German Shepherds (21%), as well as mix-breed dogs (24%) seemed to be more frequently affected.

Keywords: dog, epidemiology, hemangiosarcoma, spleen

Macroscopic changes of the splenic parenchyma can be diagnosed in veterinary practice by different methods such as: abdominal palpation, ultrasound examination, radiography or exploratory laparotomy (1). These changes can be characterized as splenic nodular lesions or diffuse lesions (splenomegaly). Splenic tumoral pathology in dogs has a high prevalence and is often diagnosed late or in the context of other primary illnesses. The most commonly used diagnostic methods of splenic neoplasia are histological examination and cytological examination (6). Hemangiosarcoma is an aggressive malignancy arising from the vascular endothelial cells, dogs being the most frequently affected among other animal species (13). Primary hemangiosarcoma can develop in any organ which benefit from vascular structures, but the most frequent sites of development include the spleen, right atrium, subcutis, dermis and the liver. Visceral localization of hemangiosarcoma is associated with a poorer prognosis and is more commonly diagnosed compared to cutaneous hemangiosarcoma (12). This tumor type metastasizes to distant organs and tissues, the lung being the most frequently affected organ. In terms of sex predisposition, the current situation is still uncertain, with no scientific data in the literature demonstrating a higher incidence of this pathology in males or females dogs.

Materials and methods

Our main purpose was to perform a retrospective epidemiological study of the past 11 years, to evaluate the incidence of malignant splenic lesions in dogs and also to describe some aspects regarding sex and breed predisposition.

For the present study we reviewed records of canine patients submitted to the Pathology Department, Faculty of Veterinary Medicine Cluj-Napoca, between April 2007 and June 2018. The study material included spleen samples harvested after splenectomy or after a necropsic examination, from canine patients diagnosed with splenic neoplasia. Gross inspection, cytological (Diff-Quick stain) and histological (H&E stain) analyses were performed. A total number of 242 canine patients met the criteria and were included in the present study. In all identified cases, the definitive diagnosis was established by histological examination and in some cases also by cytological examination (samples collected using the fine needle aspiration technique).

Results and discussion

After reviewing the records kept in the Pathology Department, Faculty of Veterinary Medicine, Cluj-Napoca between April 2007 and June 2018, we identified a total number of 1629 canine patients diagnosed with oncological diseases, out of which 6% (n=97) were diagnosed as primary splenic tumors (Fig. 1). In a research paper presented by Pastor J. in 2002 (16) it is shown that, similar to our study, hemangiosarcoma constitutes about 5% of all neoplastic diseases diagnosed in dog.

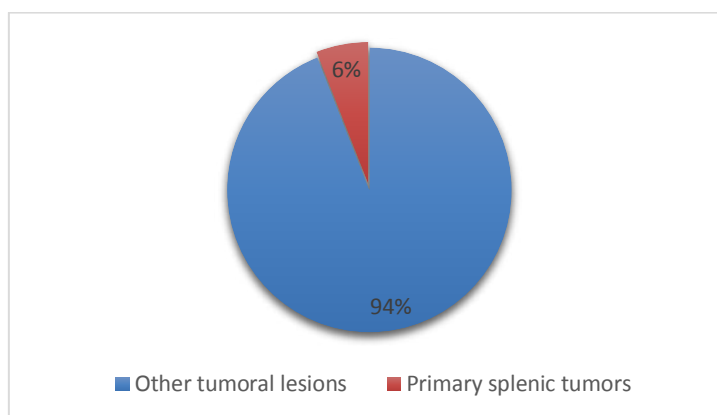


Fig. 1. Incidence of splenic neoplastic lesions

Out of all splenic lesions identified in our records, in the above-mentioned timeframe, 60% (n=145) were diagnosed as non-tumoral splenic lesions, and the remaining 40% (n=97) were diagnosed as primary splenic neoplasia (Fig. 2). By analyzing figure 3, it can be seen that from the total number of splenic tumors

diagnosed in dogs, malignant tumors represent 89% (n=86), the remaining 11% (n=11) being diagnosed as benign tumors.

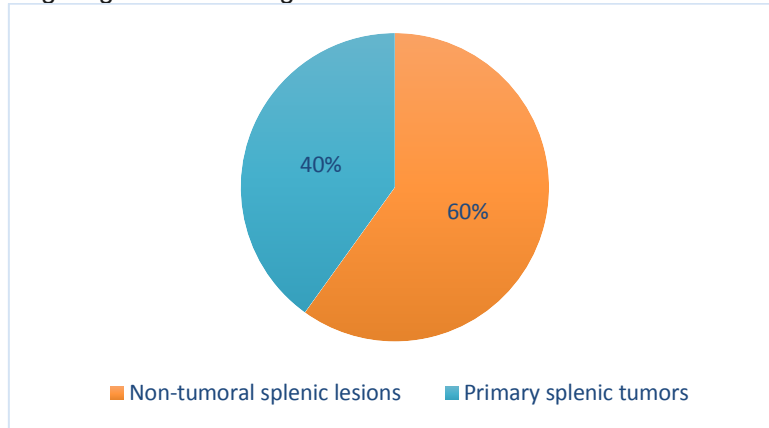


Fig. 2. Distribution of tumoral and non-tumoral splenic diseases in the dog

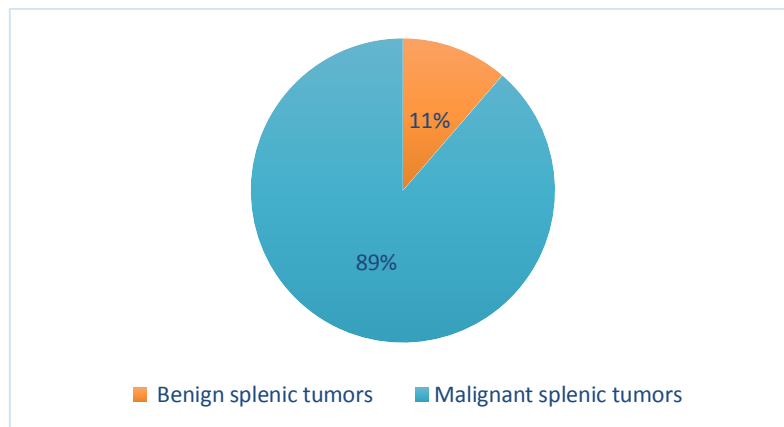


Fig. 3. Distribution of benign and malignant splenic primary tumors

In the present study, the only benign tumor encountered was splenic hemangioma (11 cases). In terms of malignant tumors, the main lesions identified in our study were represented by hemangiosarcoma (69 cases), splenic lymphoma (19 cases), histiocytoma (1 case), metastatic mast cell tumor (1 case) and metastatic osteosarcoma (1%) (Fig. 4). Between the results of the present study and the study conducted by Cleveland and Casale in 2016 (8) at the Angel Animal Health Center in Boston, there are some similarities because in the quoted study, from 105 canine patients undergoing splenectomy it was pointed out that 70.5%

were diagnosed with benign splenic tumors and just 29.5% were diagnosed with malignant neoplasia, out of which 58% were diagnosed with primary splenic hemangiosarcoma. In our study, splenic hemangiosarcoma was also the most commonly diagnosed malignant tumor, representing 75% of total splenic neoplastic lesions.

Analyzing Fig. 5, it can be seen that regarding breed predisposition, hemangiosarcoma was most frequently diagnosed in German shepherd dogs (23%), followed by mixed breed dogs (22%) , Rottweiler (11%), Cocker Spaniel (6%), German Shorthaired Pointer (5%), Poodle (5%), Boxer (5%) and Dachshund (3%).

Regarding the average age at which patients were diagnosed with hemangiosarcoma; the limits were between 5 and 18 years old, the mean being 10.91 years. Of the 64 cases diagnosed with primary splenic hemangiosarcoma, 67% of the subjects included in our study were male dogs and 33% were females. We found similarities between the present study and another epidemiologic study conducted by Clendaniel et al. in 2014 (7), which showed that out of 79 dogs diagnosed with primary splenic hemangiosarcoma, 51% were males and 49% females, the mean age of occurrence being 11.5 years and the age limits ranging from 6 to 15 years old. By studying these epidemiological data we can see that there might be a slight predisposition for male individuals, but further research is needed in order to establish a correlation between the individual's sex and the occurrence of hemangiosarcoma. Regarding breed predisposition, the same authors observed that mix breed dogs were most commonly diagnosed (35%), followed by the Labrador retriever (11%), Golden Retriever (6%) and German shepherd (6%).

Taking that into consideration the fact that hemangiosarcoma frequently metastasizes to other distant organs and tissues, we wanted to evaluate the rate of metastasis in canine patients included in the present study. We observed that 56% of the analyzed subjects presented metastases in different organs such as liver, peritoneum, lungs and intestinal serosa. The importance of the metastatic process in case of hemangiosarcoma is also demonstrated in a recent study conducted by Leyva et al in 2018 (14), which showed that out of 69 canine patients diagnosed with splenic hemangiosarcoma, 66.7% had metastases.

Aronsohn et al., in a study performed in 2009 on 60 dogs (2), identified an increased incidence of hemoperitoneum (63.3%) associated with splenic hemangiosarcoma. In the present study, out of all patients diagnosed with splenic hemangiosarcoma, only 30% developed hemoperitoneum secondary to splenic rupture. Even if the percentage obtained in our study is not as high compared to other studies, this lesion should be seriously considered, because is one of the leading death causes in patients diagnosed with splenic hemangiosarcoma

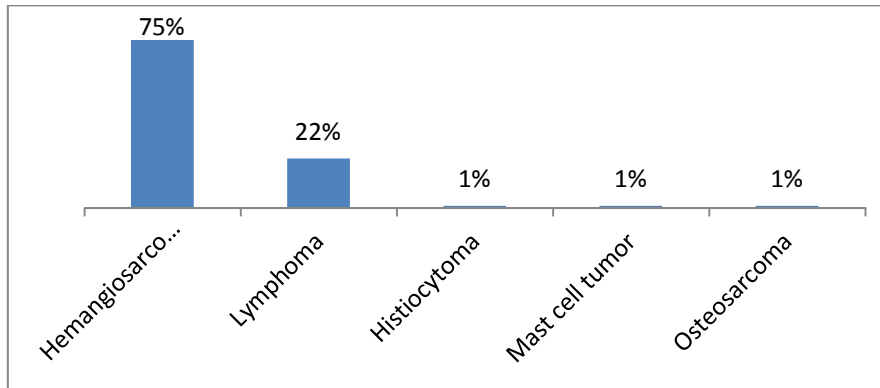


Fig. 4. Malignant splenic tumors diagnosed in the present study

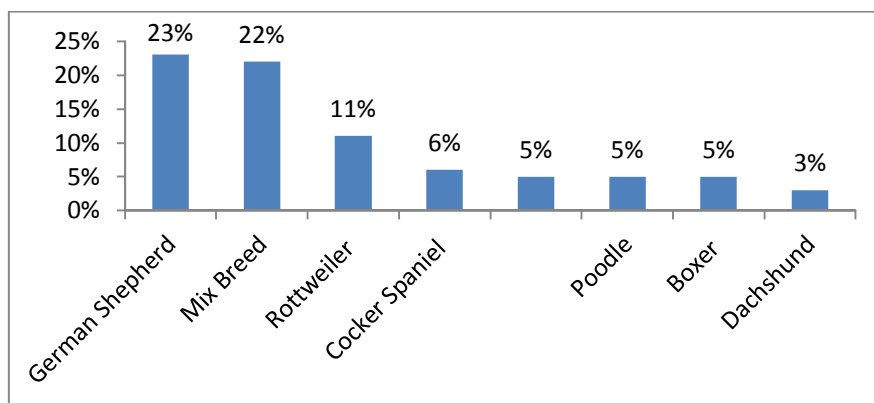


Fig. 5. Hemangiosarcoma- breed predisposition

Conclusions

Out of all tumor types diagnosed between 2007- 2018 (n=1629) in the Pathology Department, Faculty of Veterinary Medicine, Cluj-Napoca, 6% were splenic tumors. Primary splenic hemangiosarcoma was the most commonly diagnosed tumor in the spleen (75% of all cases), also with a high metastasis rate, 56% of the diagnosed subjects presented metastases in different organs. The metastasis process, related to hemangiosarcoma diagnosis is of particular importance and obliges veterinary clinicians to investigate possible metastases in different organs or tissues prior to establishing the therapeutic protocol. Regarding breed predisposition, German Shepherds (23%), as well as mix-breed dogs (22%) seemed to be more frequently affected. The average age of diagnosis for

hemangiosarcoma was 10.91 years, age limits being between 5 years and 18 years old. In terms of sex predisposition, further research is needed because in the present there are no scientific data in the literature demonstrating a higher incidence of this pathology in males or females dogs. To our knowledge, this is the first epidemiological study regarding canine splenic tumors performed in Romania.

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TESTING THE PHARMACO-THERAPEUTIC EFFICACY OF THE ALCOHOLIC EXTRACT OF *CENTELLA ASIATICA* ON THE CELL LINE HaCat

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Summary

Centella asiatica is an important medicinal herb, widely used in the Orient and popular in the West, with a broad therapeutic action that is given by the main constituents of the plant, which are the triterpenoid and saponins. The aim of this study was determination of the total and individual polyphenols content of the 10% *Centella asiatica* alcoholic extract and the evaluation of its biological and proliferative properties on the HaCat cell line, represented by human keratinocytes. For this study, the cells (keratinocytes) were divided into several batches, the extract being applied to the following concentrations: A-control, B- 5 μg / ml, C- 10 μg / ml, D- 20 μg / ml. The rate of cell proliferation expressed as a percentage, at a 24h exposure time, after application of *Centella asiatica* alcoholic extract was of 88% for the concentration of 5 μg /ml and of 87% for both 10 μg /ml and 20 μg /ml. Instead, at 48h we achieved a lower proliferation rate, of 83% for the concentration of 5 μg / ml and a proliferation rate of 84% for concentration of 10 and 20 μg / ml. The total polyphenols content at *Centella asiatica* averaged 1133.4 μg /ml with an SD of 1.3129 μg /ml and after determining the individual polyphenols at *Centella asiatica*, the most representative are: Kaempferol with the highest content of 248,209 μg / ml, Quercetin 25,001 μg / ml and Resveratrol with 20,975 μg / ml, substances with strong antioxidant properties. After studying the cellular effect and analyzing the therapeutic effect of 10% *Centella asiatica* alcohol extract on HaCat cell lines, we obtained the following: cell proliferation was observed at all doses, without dose dependence; cell growth was observed at 24h, compared with the control group; the proliferation test revealed an increase in cell development, independent of the dose administered, at both 24 and 48 hours; we did not detect any changes in the shape of the cells at 24 h exposure time, but at 48 h we observed cytoplasmic vacuoles, which suggesting lipid accumulations in the cytoplasm. Based on the research, the use of topical formula with applicability in veterinary medicine could be recommended in the treatment of various skin conditions but with further research on appropriate formulation.

Keywords: *Centella asiatica*, 10% alcoholic extract, polyphenols, antioxidants, keratinocytes

Phytotherapy is therapeutics with the active substances, biosynthesized by the plant cell. In our country, the art of healing diseases by plant has an old tradition, to which has contributed the rich flora existing on our territory, which are over 3.600 plants. *Centella asiatica* (Fig. 1) is an important medicinal plant, widely used in the Orient and popular in the West, with a broad therapeutic action that is given by the main constituents of the plant, which are the triterpenoid and saponins (5).

Centella asiatica (also known as Gotu Kola) is a perennial plant belonging to the family *Umbeliferae* (*Apiceae*), which is found throughout India, growing in wet places, at an altitude of up to 1800 m. It is found in most tropical and subtropical countries, growing in marshy areas, including parts of India, Pakistan, Sri Lanka, Madagascar, South Africa, the South Pacific and Eastern Europe (20).

The entire plant is used for medicinal purposes. It is widely used as a blood purifier, as well as for the treatment of hypertension, for improving memory and promoting longevity. In Ayurveda, it is one of the main plants for revitalizing the nervous system (5).

The dry plant contains: 0.1% volatile oil, germacrene, caryophyllene, p-cimol, pinen; flavone derivatives - quercetin glycoside, kempferol; sesquiterpene - caryophyllene, trans-farnesene, germacrene D; triterpenic steroids - sitosterol, stigmasterol; triterpenic acids - asiatic acid, 6-hydroxy-asiatic acid, madecassic acid, madasiatic acid, betulinic acid, thankunic acid, isothanic acid; saponins or pseudosaponins (1-8%) - asiaticozide (major component), asiaticozide A, asiaticozide B, braminoside, brahmoside, brahminoside, thankuniside, isothankuniside. These triterpenic saponins and sapogenins are primarily responsible for wound healing and vascular effects by inhibiting collagen production at the site of the wound (2). The leaves are rich in carotenoids, vitamins B and vitamin C (19).



Fig.1. *Centella asiatica* (23)

Starting from the consideration that the pharmacological and therapeutic properties (Table 1) of a species are given by their chemical components or the so-called active principles specific to each species, we determined the total and individual polyphenol content of the 10% *Centella alcoholic* extract and evaluate its biological and proliferative properties on the HaCat cell line. The objectives of the study were to determine individual polyphenols by LC-MS and *in vitro* evaluation of cellular effects and to analyze the therapeutic effect of 10% *Centella asiatica* extract on the HaCat cell line.

Table 1

Pharmacological activity by types of *Centella asiatica* extraction

Pharmacological activity	Type of extract	Laboratory animals used	References
Hepatoprotective	Powder from the whole plant	Rats	4
Anticonvulsant	Hydroalcoholic extract Ethanol extract	Albino mice and rats Mice	9
Imunomodulator	Methanolic extract	Mice	11
Antimicrobial	Ethanol extract	Mice	14
Antipsoriatic	Aqueous extract and saponosides triterpenes	Keratinocytes in vitro	17
Sedative	Hydroalcoholic extract Ethanol extract	Mice. Male Wistar rats.	8
Induces changes in gene expression	Triterpenes	Normal human fibroblast cell lines	3
Cytotoxic and antitumoral	Crude extract	Skin tumors of mice	1
Antispasmodic	Alcoholic extract	Rats	6
Antiviral	Aqueous extract	anti-herpes simplex	21
Antifilarial	Ethanol extract	Dog infestation with <i>Dirofilaria immitis</i> .	18
Immunostimulant	Water suspension	Rats	11
Antiinflammatory	Aqueous extract	Rats	15

Materials and methods

We weighed 10 g of *Centella asiatica*, which were macerated according to the instructions of the *Romanian Pharmacopoeia* X-th Edition, for 10 days, stirring three times a day in 100 ml alcohol of 70 (v / v) in brown bottles. After extraction and pressing of the residue, the extractive liquid was left to sediment at 5-10° C for six days after which it was filtered (22).

After filtration, some of the extract was lyophilized in the Horia Cernescu Research Laboratory Complex, the analyzes being carried out in the process of validate the method of lyophilization of the vegetable matrices (29). All weighings were carried out using the Kern analytical balance.

Fig. 2. Alcoholic extract of *Centella asiatica*

For freeze-drying, the Ilshin Kryptonstraat 11, 6718 WR EDE lyophilizer (temperature -55°C , 5mTorr pressure, lyophilization time 24 hours) we used (29). The lyophilization yield of *Centella asiatica* extract is shown in Table 2.

Table 2

The lyophilization yield of the sample

ID sample	Initial quantity g	Final quantity g	Lyophilization yield (%)
2/2	31,7052	1,1217	3,54

Determination of total polyphenols was performed by the Folin Ciocalteu method on a UV-VIS Perkin Elmer Spectrophotometer, Lambda 25, software version Lambda 25.1.27, on the 190-1100 nm range, 240 nm / min scanning speed, at one nm steps (31).

To determine the individual polyphenols of *Centella asiatica* alcoholic extract, a Shimadzu chromatograph equipped with I SPD-10A UV and LC-MS 2010 detector, EC 150/2 Nucleodur C18 Gravity SB 150 x 2mm x 5 μm column we used (30).

Chromatographic conditions were as follows: mobile phase A: water acidified with formic acid at pH-3, B: acetonitrile acidified with formic acid at pH-3, gradient program: 0.01-20 min. 5% B, 20.01-50 min 5-40% B, 5-55 min, 40-95% B, 55-60 min 95% B. Solvent flow rate 0.2 ml / min, temperature 200°C . The monitoring wavelength was 280 nm and 320 nm. Calibration curves were performed in the range 20-50 μg / ml (30).

The pharmaco-therapeutic activity of *Centella asiatica* alcoholic extract were tested on the HaCat cell line (keratinocytes).

We prepared a 25 cm^2 flask containing five-seven ml of complete medium, then remove the cryotube from the cell bank (ultra-freezer -150°). After three-six hours, when the cells adhered, the culture medium was changed to a fresh one. This is necessary because DMSO used for freezing as anti-crystallizing is toxic to cells (24).

The cells were cultivated in DMEM culture medium supplemented with 10% FCS (fetal calf serum) and 1% Penicillin / Streptomycin and incubated at 37°C and 5% CO_2 . At the time they became confluent, the HaCat control cultures were further expanded by performing three complete 1:4 complete passages by harvesting the adherent cells. Cell extraction was performed by treatment with Trypsin / EDTA after a preliminary washing with PBS. After trypsinization, cell viability and total cell count were determined (25).

Determination of viability is performed by the 0.4% Trypan Blue Exclusion Test. Dead cells lose the integrity of the membrane and will stain in blue, and the living ones will remain uncolored, shining on a blue background (26).

We prepared a 1:1 dilute cell suspension in the dye and loaded 10 μL into the Neubauer counting room. For the Neubauer room, the room depth is 0.1 mm,

of which 1 mm² is used and the volume is 0.1 mm³. There are both counting, live cells (uncolored) and also dead (colored) ones (26).

Multiple counts are performed in the five quadrants and media is performed (Fig 3).



Fig. 3. Neubauer counting cell - live cells (translucent white) and dead cells (blue)

$$\% V = \frac{\text{no. viable cells}}{\text{no. total cells}} \times 100 \quad (26)$$

(percentage of viability)

$$\text{Cell density} = A \times B \times C \times 10^4,$$

A – cell volume (10 μl);
B – the dilution factor;
C- number of cells ;
10⁴ – the corection factor;

After counting, the cells were resuspended in the medium to perform the assays. For the MTT test cells were seeded in 96 well plates, in number of 2 x 10⁴. Add 10 μL of 12 mM MTT in each well plates and incubate at 37 °C for four hours. After incubation, the medium is removed, keeping only 25 μL in each well. 50 μL of DMSO (dimethylsulfoxide) is added and incubated at 37° C for 10 minutes. Read absorbance at 540 nm. MTT reduction is expressed as a percentage of the control value (27).

The lyophilized extracts were suspended in specific culture medium to obtain a stock solution. From the stock solution, solutions of different concentrations for the test were prepared by diluting in the medium. The cells were divided into several batches, the extract being applied to the following concentrations: A. control B. 5 μg / ml C. 10 μg / ml D. 20 μg / ml.

The effect of Centella asiatica extract was evaluated by cell proliferation / multiplication assay, estimating the rate of proliferation of the HaCaT keratinocyte cell line. Cell proliferation was determined using the Vybrant MTT Cell Proliferation Assay Kit (Invitrogen), which is a simple method for determining the number of cells using the standard absorbance reader kit (28). Cellular morphology was highlighted by MCF (phase contrast microscope) and allowed to identify the shape of the cells.

Results and discussions

The total polyphenol content of the 10% *Centella asiatica* alcoholic extract was 1133.4 $\mu\text{g/ml}$ (Folin Ciocalteu method) according to Table 3, with the UV-VIS spectrum shown in Fig. 4.

Table 3

The total polyphenol content

ID sample	Content in polyphenols ($\mu\text{g/ml}$) Repeat 1	Content in polyphenols ($\mu\text{g/ml}$) Repeat 2	Content in polyphenols ($\mu\text{g/ml}$) Repeat 3	Content in polyphenols ($\mu\text{g/ml}$) Mean	Content in polyphenols ($\mu\text{g/ml}$) SD
2/2	1132,3	1133,1	1134,9	1133,4	1,3129

Fig. 4. UV-VIS spectrum of *Centella asiatica* extract

Following the determination of individual polyphenols by LC-MS, the results obtained are presented in Table 4 and Fig. 5.

Table 4

Quantitative results – chromatogram *Centella asiatica* extract 10%

Standard no.	Name	<i>Centella asiatica</i> ($\mu\text{g/ml}$)
1.	Gallic acid	3,506
2.	Proto catehuic acid	1,767
3.	Caffeic acid	1,144
4.	Epicatechin	0
5.	p- coumaric acid	0
6.	Ferulic acid	8,845
7.	Rutin	32,420
8.	Rosmarinic acid	18,534
9.	Resveratrol	20,975
10.	Quercitin	25,001
11.	Kaempferol	248,209

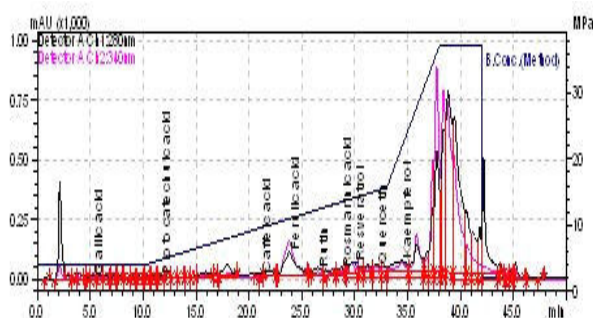


Fig. 5. Chromatogram of *Centella asiatica* alcoholic extract

MTT Cell Proliferation Assay Results

Cell proliferation was highlighted at all doses, without dose dependence. After the application of the extract, the increase in cell multiplication was observed at 24h compared to the control group (Fig. 6), as indicated by the MTT assay (Table 5). The proliferation assay revealed an increase in cell growth independent of the dose administered at both 24 and 48h (Fig. 7).

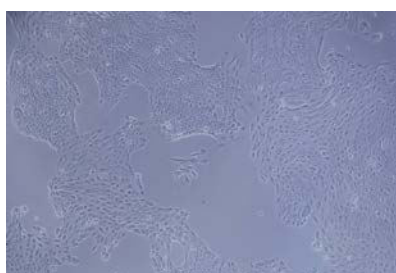


Fig. 6. HaCat cells control batch, MCF, 400x

Table 5
Cell proliferation rate (%) following application of *Centella asiatica* alcoholic extract

Proliferation rate (MTT) (%) at 24 h	Proliferation rate (MTT) (%) at 48 h	Concentration
88	84	5 µg/ml
87	83	10 µg/ml
87	83	20 µg/ml

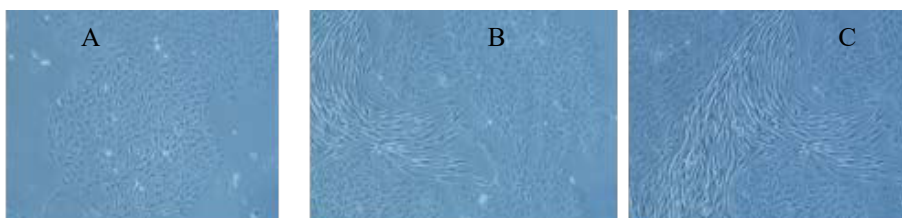


Fig. 7. HaCat cells dose 5 µg (A), 10 µg (B), 20 µg (C) / ml at 24 h after exposure MCF, 400x

Analyzing cell morphology, we did not see any changes in the shape of the cells at a 24-hour exposure time.

At 48h hours after exposure, we observed cytoplasmic vacuolization, suggesting lipid accumulations in the cytoplasm (Fig. 8).

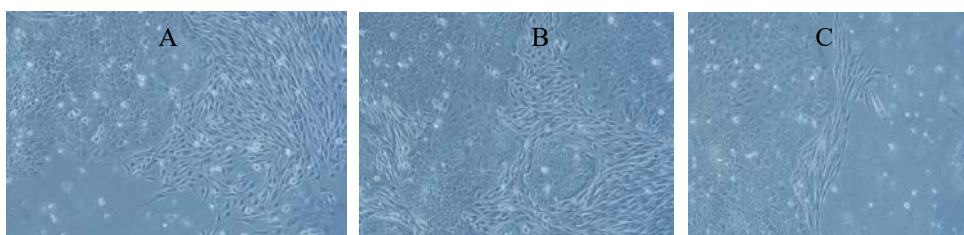


Fig. 8. HaCat cells dose 5 µg (A), 10 µg (B), 20 µg (C) / ml at 48 h after exposure, MCF, 400x

The most common mammalian connective tissue cells are fibroblasts, which play a critical role in wound healing. In the case of damaged tissue, fibroblasts are those that proliferate, migrate to the wound and produce large amounts of collagen matrix, matrix that helps isolate and restore damaged tissue. There are authors who claim that *Centella asiatica* is effective in treating wounds, even infected, burns and postoperative hypertrophic scars (12, 13).

Triterpenic compounds (asiatic acid, madecassic acid, asiaticozide and madecassoside) are the main components of *Centella asiatica* plant responsible for wound healing. The action has been demonstrated for both, extracts and triterpene compounds in a large number of scientific studies in which *in vitro* and *in vivo* research have been conducted. Terpenoids are the ones that cause a significant increase in the percentage of collagen and fibronectin in the cell layer. The most beneficial effects are stimulation of scar maturation by the production of type I collagen, decrease of inflammatory reaction and production of miofibroblasts (7).

Aqueous extracts from *Centella asiatica* also exhibit anti-psoriatic activity and exert a beneficial effect in treating systemic scleroderma and focal scleroderma (10).

Chromatographic analysis of *Centella asiatica* extract revealed large amounts of kaempferol, quercetin, rutin, resveratrol and rosmarinic acid. Kaempferol is a powerful antioxidant and helps prevent oxidative lesions of cells, lipids and DNA. Kaempferol appears to prevent arteriosclerosis by inhibiting the oxidation of low density lipoproteins and blood platelets. Studies have also confirmed that kaempferol acts as a chemopreventive agent, which means that it inhibits the formation of cancer cells (16).

The kaempferol and quercetin flavonoids seem to act synergistically in reducing cancer cell proliferation, which means that the combined treatments with quercetin and kaempferol are more effective than the additive effects of each flavonoid (16).

Conclusions

Based on the 10% *Centella asiatica* alcoholic extract at concentrations of 5, 10 and 20 $\mu\text{g} / \text{ml}$, using the HaCaT cell line, at 24 and 48 hours post-exposure and using the MTT assay, the following were observed: increased cell development, independent of the dose administered, at both 24 and 48 hours; at 24 hours after exposure there is no change in the shape of the cells; at 48 hours after exposure, cytoplasmic vacuolization was observed, suggesting lipid accumulations in the cytoplasm; chromatographic analysis of the *Centella asiatica* alcoholic extract revealed a series of polyphenolic compounds, the highest concentrations being kaempferol, quercetin, rutin, resveratrol and rosmarinic acid, substances with strong antioxidant properties.

Following research, it may be advisable to obtain and use topical formula with applicability and in veterinary medicine to treat various skin conditions, but additional research is needed because finding a suitable formula is a real challenge.

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SPINAL CORD INJURY IN DOGS: A RETROSPECTIVE STUDY

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Summary

The most common spinal cord injury in dogs is the Intervertebral disc disease type 1 or 2. It is known that two types evolve differently depending on breed and age. The aim of this study is to assess the incidence of clinical syndrome in patients with spinal syndrome between years 2015 and 2018. To conduct this research we used the medical data registered in the Faculty of Veterinary Medicine Iași. From 524 neurological patients, 118 were included in this study and further analyzed for the following factors: breed, sex, age and neurolocalization of spinal cord syndrome. Thoraco-lumbar syndrome present in 50% of cases had the highest incidence, while lumbo-sacral syndrome was constituted only 11%. The results indicate that mongrels represented 56% of patients with spinal cord syndrome followed by Bichon with 29%. Beagles are susceptible for cervical syndrome (15% of all cases). Bichon breed 12% for the cervico-thoracic region. Mongrels represented 56% for the thoraco-lumbar region and 17% for the lumbo-sacral region. Analyzing sex, the following data was obtained: 76% of males had injuries in the cervical region, 55% females in the cervico-thoracic region, 51% of females in the thoraco-lumbar region and of 60% females in the lumbo-sacral region. Patients aged less than 6 years and over 6 years had approximately an equal incidence on thoraco-lumbar syndrome, 57% and 58%. Based on this data, we can conclude that breeds such as Beagle are prone to the pathology of cervical syndrome, Bichon to cervico-thoracic syndrome and mongrels to thoraco-lumbar and lumbo-sacral syndrome. Thereby, could create an algorithm and use it for differential diagnosis.

Keywords: dog; spinal cord injury; spinal syndrome

Neurological pathologies are quite common, accounting for 11% of all patients presented to the Faculty of Veterinary Medicine, Iași, at the Clinic of Internal Medicine in the last 3 years (2015-2018). Of these, 18% are patients with vertebral lesions. The most common vertebral lesion is represented by intervertebral disc herniation and trauma by luxation or fractures of the vertebral body.

The aim of this study is to characterize the epidemiological situation of the spinal pathology in each spine region of the Internal Medicine clinic of Faculty of Veterinary Medicine, Iași.

Materials and methods

The inclusion criteria were represented by the dogs of various ages (<1 years – 18 years), breeds and sex categories, neurological deficits due to spinal cord injuries and no other intracranial pathologies (based on normal consciousness

and normal cranial nerves). Each dog underwent a neurological examination in the clinic and the diagnosis was made by radiography and myelography. All data concerning age, sex, breed and affected spinal regions were recorded in the electronic archive of Marasoft program.

Subsequently, all information recorded between 2015-2018 was retrieved from the faculty clinic database. The patients included in this study were grouped by breed, sex and age. Depending on age, the subjects were divided into two categories. The first category represented dogs under the age of 6, which included young and adults and the second one included patients over the age of 6 year. Data was analyzed in Microsoft Excel through descriptive statistics.

Results and discussions

According to our data, during the 2016, 2017 and 2018 period, were registered 524 dogs with neurological diseases, of which 118 had a spinal pathology. The thoraco-lumbar region was frequently affected, with 50% of lesions at this spinal level. 27% of the lesions were recorded at the cervical level, 12% cervico-thoracic and 11% lumbo-sacral (Fig. 1).

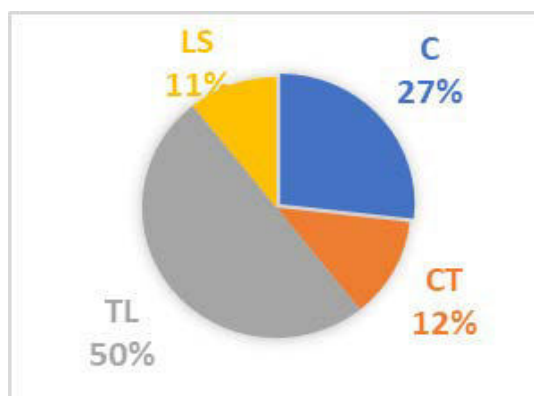


Fig.1. Percentage of distribution in medullary syndromes

The small sized dogs were often affected. In this category were included the following breeds: Shi-tzu, Teckel, Westie, York Shire, French Bulldog, Caniche, Chihuahua, Fox Terrier and Jack Russell Terrier. Out of those, the most affected were Bichon (18%) and Pekingese (10%). 21% represented the rest of the affected breeds. 35% were mixed-breeds of small size. Large breeds with spinal disorders accounted for 16% of the total subjects studied. In this category were included dogs of the following breeds: Rottweiler, Setter, Boxer, Pointer, German Shepherd, Carpathian Shepherd, Spanish Cocker, Labrador, Amstaff and Beagle (Fig. 2).

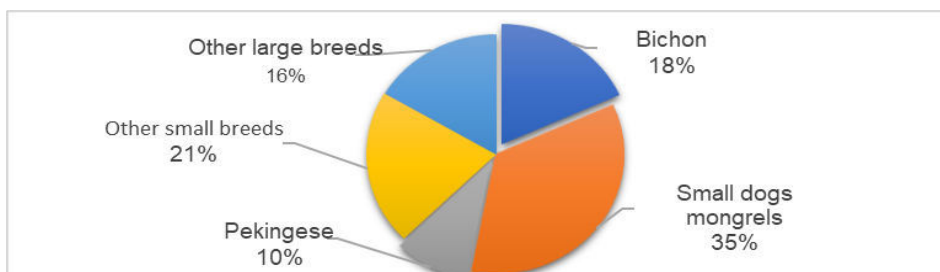


Fig. 2. Distribution of patients by breeds

Thoraco-lumbar region is one of the most common spinal segment affected in dogs (6, 5, 7). The results obtained by us were similar with those of S.A. Moore, where the most affected area was also the thoraco-lumbar region, representing 87% of the total dogs studied (3).

The number of small sized breed dogs (n. 90) affected by spinal lesions was higher in comparison to the larger ones (n. 28). Our results can be explained by the fact that geographically, small sized dogs are more common. The results obtained by us accounted for 35% of the subjects being small half breeds. It should be noted that those dogs were mixed-breeds by Teckel, Pekingese Bichon, Westie, York Shire, etc. However, small breed dogs can present a genetic predisposition for degeneration of the intervertebral disc. An expressed fibroblast growth factor (FGH) - 4, was shown to be associated with intervertebral disc disease in these breeds. In another study, Jonathan M. Levine argues that Teckel pedigree breeds carry an autosomal polygenic gene that induces intervertebral disc herniation (1, 3, 4).

The first category of age affected by spinal lesions (under 6 years old) accounted for 59% of the total, and the most common age with spinal disorders from this group was at 4 and 5 years old (15% / 12%). The second category of age (above 6 years old) represented 41% of the total and this one included older and geriatric (41%), the most affected age being 9 year (9%) (2, 3, 4).

Both categories had more frequent lesions in the thoraco-lumbar region (57% under 6 years and 58% over 6 years). For the category below 6 years old we obtain that 24% of subjects had lesions in cervical region, 11% in cervico-thoracic region and 8% in lumbo-sacral. For the group over 6 years old: 30% cervical region, 6% cervico-thoracic region and 6% lumbo-sacral (Fig. 3, 4).

Our result show that the young and middle-aged adults (under 6 years old) represented the highest affected group by spinal diseases. The increased incidence of spinal pathologies was at the average to 5 years old (15%) from this category of the total subjects studied. Our results were almost similar to those of S.A. Moore and Jonathan M. Levine in their studies. S.A. Moore affirm that the

average age of subjects with spinal pathology was 6 years old and 90% of them were small dogs (under 20 kg). Jonathan M. Levine had the same result that the most common category affected is represented by youth and middle-aged dogs (4).

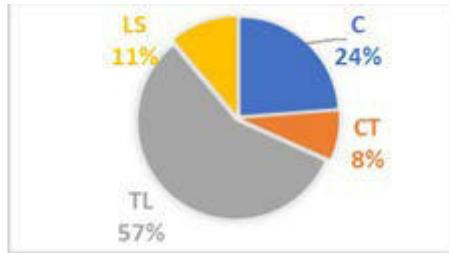


Fig. 3. Under 6 years old with spinal injury in medullary regions

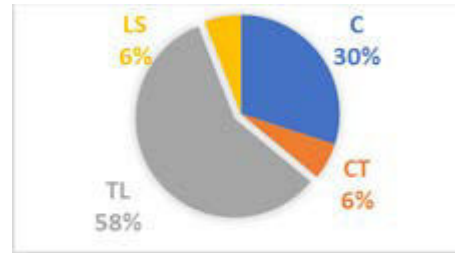


Fig. 4. Over 6 years old with spinal injury in medullary regions

Based on the data interpretation of the total population surveyed, 58% of affected dogs were females (F) and 42% males (M) (Fig. 5).

For every spinal region affected, we obtained: on the lesions of the cervical region (C1-C5) - 76% were males and 24% females, in the cervico-thoracic region (C6-T2) - 45% were males and 55% females, in the thoraco-lumbar region (T3-L3) - 49% were males and 51% females and in the lumbo-sacral region (L4-S3) - 40% were males and 60% females (Fig. 6, 7, 8, 9).

For the cervical region, the frequency of spinal injuries was higher in males representing 76% of all dogs with pathology in the C1-C5 region. The rest of the regions, respectively C6-T2, T3-L3 and L4-S3, showed that lesions appear frequently in males. Our results about the increased number of females with spinal disorders was different with those of S.A. Moore from his article in which the incidence of spinal diseases was higher in males with 54% and 46% in female (4).

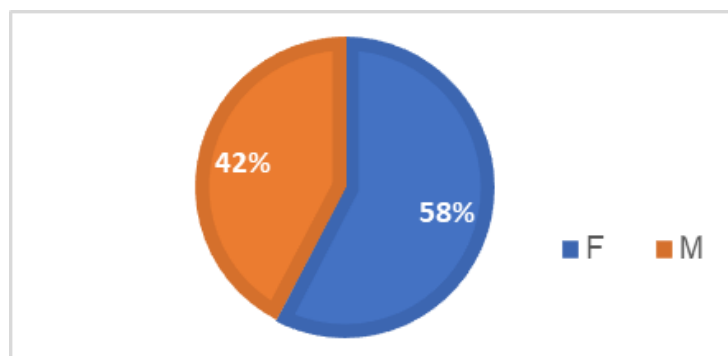


Fig. 5. The number of patients depending on sex

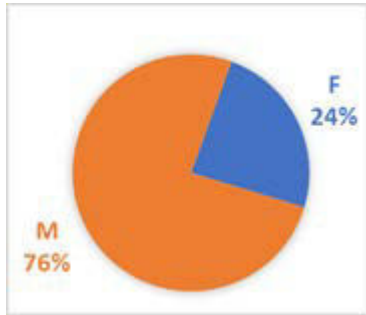


Fig. 6. Distribution by sex region C1-C5

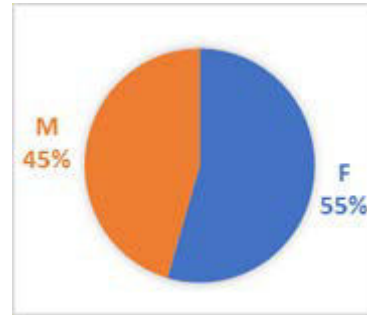


Fig. 7. Distribution by sex region C6-T2

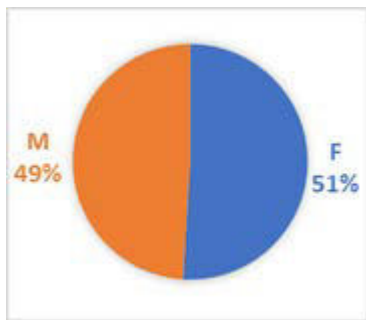


Fig. 8. Distribution by sex region T3-L3

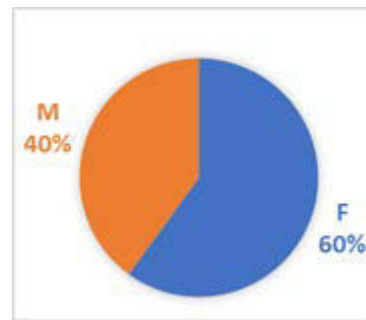


Fig. 9. Distribution by sex region L4-S3

Conclusions

The T3-L3 spinal segment represent the most common region affected by injuries from the total population of this study. Small sized breeds and their mongrel are often affected by spinal pathology. The average of 5 years old represent the category of age affected by spinal disease in our study. The incidence of the spinal cord disease was higher in female patients, with the only exception being the cervical region where more male dogs were recorded.

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RESEARCH ON ALLERGIC DISEASES IN DOGS

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Summary

In this study we tried to identify the allergens with the highest potential in triggering allergies in dogs in the western area of Romania. Through the Polycheck[®] test, 32 dogs with allergic symptoms aged between 6 months and 8 years were tested. We mention that the period, considered critical in triggering allergic conditions in dogs, is around the age of 3 years. Test dogs are part of breeds with predisposition for allergies and mix breeds. The tests concluded that the main allergens involved in triggering allergic symptoms were mites, of which *D. farine* was involved in 26 out of 32 cases (81.2%), *Acarus siro* in 13 cases (40.6%), *D. pteronyssinus* in 10 cases (31.2%), *Lepidoglyphus* in 7 cases (21.8%) and *Tyrophagus* in 4 cases (12.5%). Only values that exceeded 2kU / L were considered, these being considered with increased reactivity. Other allergens that ranged above 2kU / L were represented by the different types of pollen (rye, ragweed, plantain, birch, sorrel, parietaria) in proportion of 21.8%. Based on the results, dog desensitization therapy with positive results was attempted in 5 of the 32 dogs, the rest requiring continued treatment along with desensitization.

Keywords: dogs, allergy, allergy tests (Polycheck[®])

In recent years there has been an exponential increase in the number of canine patients with allergic symptoms. This is due to a combination of factors, the genetic factor is the first factor and it is compelled by environmental factors and immunological disorders (1).

The term allergy means an immune reaction that expresses different energy than normal reactions after secondary exposure to an antigen. Allergy is currently defined as a state of hypersensitivity resulting from exposure to an allergen and is distinguished by the overproduction of immune components. Hypersensitivity states are a consequence of the fact that the immunization process after primary contact with the antigen and the generation of immune effectors (antibodies and effector lymphocytes) does not always confer a favorable state of resistance to the organism. Primary contact with the antigen sometimes creates an awareness of the antigen. Sensitization is a physiological state detrimental to the body and is manifested, especially after contact of the organism with protein antigens (egg, serum) with antigens in the pollen and less frequently after contact with the corpuscular antigens (2).

The term "allergen" is used to define the antigenic substance that induces the production of specific IgE antibodies (4).

Allergy can be manifested clinically in the form of several entities: contact dermatitis, allergic otitis, allergic rhinitis, asthma. There are a multitude of allergens that can trigger clinical manifestations among the most common are mites, pollen, molds, pollutants (3)

Because of the multitude of allergens and the impossibility to control exposure to these allergens, we tried to find a quick test to help identify the major allergens in an attempt to provide a more accurate therapy to patients.

Materials and methods

Thirty-two privately owned dogs participated in this study, which took place in Timișoara between 2014 and 2018. All dogs were diagnosed with atopic dermatitis based on both clinical signs and medical history. Other dermatological problems such as food allergic reaction, ectoparasites or endocrine diseases have been excluded. Any kind of treatment was discontinued two weeks before the allergic test.

The dog breeds included in this study were American Staffordshire Terrier (n = 1), Labrador Retriever (n = 1), Yorkshire terrier (n = 1), Teckel (n = 2), Shih-Tzu (n = 2), Jack Russell Terrier (n = 1), Pekingese (n = 1), West Highland White Terrier (n = 4), Maltese (n = 1), French Bulldog (= 1), White Swiss Shepherd Dog (n = 1), Mix breeds (n = 11).

Ten out of thirty-two dogs were females (31,25%) and twenty-two were males (68,75%). Eleven dogs from thirty-two were sterilized (34.3%). The age of the dogs was between 6 months and 8 years with the average age of 3 years.

The Polycheck® test was purchased from Biocheck GmbH Laboratory in Germany with the ability to detect the following allergens: *Dermatophagoides farine*, *D. pteronyssinus*, *Malassezia*, *Lepidoglyphus spp*, *Aspergillus/Penicillium*, *Alternaria/Cladosporium*, ragweed, birch/alder/hazel, platane/willow/poplar, parietaria, rye pollen, grass-mix, stinging nettle, lambs quarter, plantain, muqwort, sorrel, *Acarus siro*, *Tyrophagus*, flea (*Ctenocephalides*). Immunoassays were performed according to the manufacturer's protocols in the kit.

The tests are done with serum, the blood was collected in coagulation activator tubes, 30 minutes after blood collection, the sample was centrifuged for 3-5 minutes at 3000 rpm. After centrifugation the expressed serum was transferred to Eppendorf tubes and kept in the refrigerator at 2-8 °C until processing. A detection set contains 12 kits. The detection kit contains allergen detection solutions, a buffer that must be reconstituted with distilled water and kept in the refrigerator until use, and the reaction plates next to the frame. All solutions are brought to room temperature before use.

For each serum, a plate is placed on the frame, marked with a corresponding serum marker.

The first step requires placing the plates on a 30 rpm shaker following the washing of the plate with 250 µl of wash buffer and excess liquid removal by pressing multiple times on an absorbent paper.

Then addition of 250 μ l of the starting solution from the set and maintaining for 5 minutes at 30 rpm, followed by removal of excess liquid by the same procedure. 200 μ l of serum is added and kept for 60 minutes at 30 rpm and then washed with 1 ml of distilled water three times, and then the excess liquid is absorbed.

The procedure continues with 250 μ l of wash buffer for 5 minutes and removing excess solution, again washing with distilled water 1 ml of solution 3 times. The washing step with buffer solution and distilled water was repeated twice.

In the following step 250 μ l anti-IgE antibody solution is added and kept for 90 minutes, then washed with distilled water 1 ml 3 times.

Followed by enzyme-labeled anti-ligand solution 250 μ l for 20 minutes and washed with distilled water. 250 μ l substrate solution is added for 20 minutes, this reaction will take place in the dark, requiring coverage of the kit and washing with distilled water. Dry the kit for 15 minutes.

After complete drying, the plates will be read using a scanner and Biocheck[®] program.

Results and Discussions

The percentage of positive reactions for each group is shown in Fig. 1. Nine out of thirty-two dogs (28.1%) responded to a single allergen with values above 2 kU/L, considered to be highly reactive, the rest of the animals reacted to two or more allergens.

The highest percentage of reactivity was against mites (84.3%). *D. farin* was involved in 26 out of 32 cases (81.2%), *Acarus siro* in 13 cases (40.6%), *D. pteronyssinus* in 10 cases (31.2%), *Lepidoglyphus* in 7 cases (21.8%) and *Tyrophagus* in 4 cases (12.5%).

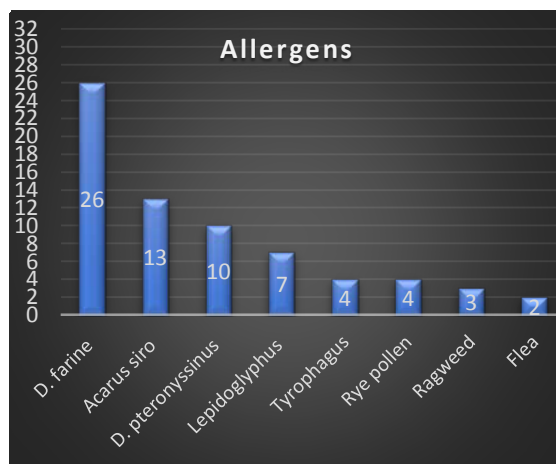


Fig.1. Number of positive cases and the allergens with the highest prevalence

Seven out of thirty-two dogs reacted to the pollen: Rye pollen (9,37%), Ragweed (6,25%), Grass-mix (3,12%), Plantane (3,12%). Two of 32 dogs reacted to fleas (6.25%).

In this study, small breeds were the most affected, two of the breeds were more affected West Highland White Terriers (12.5%), Maltese (9.37%). Small breeds are preferred because they are easier to maintain in homes, but the lack of adequate and closed spaces are the cause of triggering allergies, especially those caused by mites.

Mites are involved in most of human and other species allergies. There has been an increase in the number of atopic dermatitis due to ambrosia pollen, the prevalence of this allergy in the Romanian population is 5.35%, which indicates that measures to prevent the spread of this plant are unsuccessful (5).

Allergen specific immunotherapy could offer a more definitive treatment and should be recommended when the avoidance of allergens is impossible (3).

Conclusions

Our investigation found that the most common causative allergens are mites followed by pollens. Molds do not determine as much allergens in Romania compared to studies done in other countries on dog allergies. The climate changes might reflect the findings along side the pollution and the indoor maintenance of the dogs.

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ANTIBACTERIAL PROFILE OF STAPHYLOCOCCAL ISOLATES ASSOCIATED WITH THE BUBALINE MASTITIS

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Summary

Between January and May 2017, 68 samples of milk were collected from bubalines reared for milk production in the south-western part of Arad County, Pecica, in a herd summing a total of 43 Bubalines. The samples were examined using indirect tests for the detection of mastitis, California Mastitis Test. From the positive tests to indirect tests, 38 samples of milk were processed for the bacteriological examination and antibiogram. The identification of the bacterial agents was realized according to standard methodology by studying the cultural, morphological and biochemical characters. The antibiotic susceptibility test was performed using Kirby-Bauer diffusion method using the following antibiotics: methicillin, ampicillin with sulbactan, tetracycline, doxycycline, gentamycin, kanamycin, erythromycin, vancomycin, ciprofloxacin, polymyxin B, novobicin, rifampicin, ceftrioxone, ceftioxin, cefaclor. Following the evaluation of the milk samples, 26 strains of staphylococcus were have been isolated, 19 of the coagulase-positive (*S. hyicus* and *S. aureus*) and 7 coagulase-negative strains (*S. haemolyticus*, *S. sciuri* and *S. epidermidis*). The antibiotic susceptibility of these staphylococcus strains isolated from the mastitic milk was variable depending on the antibiotic groups. For the β -lactams used (methcillin, ceftriaxone, ceftioxin, cefaclor, ampicillin with subactan), the antibiotic sensitivity was maximal, except methcillin where resistant strains were isolated from. All the isolated strains were resistant to polymyxin B, and sensitive to ciprofloxacin.

Keywords: bubaline, mastitis, Staphylococcus, antibacterial

Staphylococci, and in particular *Staphylococcus aureus*, are significant bacterial pathogens associated with bubaline mastitis. Over the past decade, infections associated with methicillin-resistant *S. aureus* (MRSA) have been described in several animal species, including bubaline.

Materials and methods

It has been taken into account a farm belonging to a household, from the south-western part of Arad county, in Pecica town in which buffalos were grown for dairy production, amounting a total of 43 buffalos of common breed, in which indirect diagnostic tests of clinical and subclinical mastitis were performed with R-Mastitest. The experiment was carried out over a period of 5 months, between January and May 2017. A number of 68 samples of milk were taken; all of these samples were examined by indirect mastitis detection tests using the California

Mastitis Test (3). 38 positive reaction samples were taken and after that were processed and prepared for the bacteriological examination, and for the antibiogram.

For the bacteriological examination the milk samples were processed in the Laboratory of Research on Bacterial Infectious Diseases (B.6.e) from the Department of Infectious Diseases and Preventive Medicine of the Faculty of Veterinary Medicine Timisoara.

The identification of the bacterial agents in the milk samples involved the study of the cultural, morphological and biochemical characteristics of the isolated bacterial agents, including the following steps:

- Sampling;
- Direct bacterioscopic examination of the milk sampled;
- Isolation of the bacterial agents in pure culture;
- Typification of the isolated bacterial agents by establishing the morphological, cultural, and biochemical characters.

In order to obtain accurate results, from a bacteriological point of view, the sampling was done under special care, so as to be less exposed to contamination with environmental bacteria, in the following way:

- The udder and the mamelon have been washed and dried well with paper towels;
- The containers used for sampling were pre-sterilized. After the containers were closed, each sample got a serial number;
- At the time of harvesting the containers were held in near-horizontal position to avoid contamination of the milk sample with impurities from the skin, or hair of the examined cow;

To isolate the bacteria in pure culture the milk samples have been seeded on liquid culture (broth) or solid culture (enriched with 5% sterile bovine blood).

The insemination of the milk on the respective media was performed under aseptic conditions, under the protection of the flame from the gas lamp, using sterile instruments (Pasteur pipettes, and bacteriological loops) and containers (Petri dishes and plates) with sterile culture media.

After 24 hours of incubation the samples were examined. From the colonies existing in the Petri dishes and in broth Gram stained smears were performed to reveal some of the morphological characters of the bacterial agents (form, grouping, etc.). Purification of the bacterial etiological agent was accomplished by replicating a single colony using the bacteriological loop and passing in onto 5% agar with blood.

Subsequently, after this partial identification and purification the typification of the isolated bacterial agents was made by the determination of metabolic and biochemical characters.

Initially, for each culture two types of reactions or tests were studied evidencing the presence of oxidizing or reducing enzymes, namely oxidase and

catalase. These tests indirectly differentiate the genres: *Staphylococcus spp.* (which are oxidase-negative and catalase-positive, except *Staphylococcus aureus*, subspecies *anaerobius*), *Streptococcus spp.* (which are oxidase and catalase – positive).

After performing these tests, for further study of the biochemical characters of the isolated bacteria and for their taxonomic classification microtests were used.

For the taxonomic classification of the isolated bacteria in milk the API Staph tests, produced by BioMerieux SA, France, were used.

The antibiotic susceptibility test for the isolated staphylococci strains was performed using the Kirby-Bauer diffusion method using the following ingredients: Mueller-Hinton broth and agar, Petri plates and biodisks impregnated with antibiotics, produced by Oxoid company. The following antibiotics were used: methicillin, ampicillin with sulbactam, tetracycline, doxycycline, gentamycin, kanamycin, erythromycin, vancomycin, ciprofloxacin, polymyxin B, novobiocin, rifampicin, pristinamycin, lincomycin, ceftriaxone, ceftiofur, and cefaclor. The antibiotic susceptibility test was also performed in order to establish the therapeutic conduit to the respective clinical cases, to identify the methicillin resistant strains, and to identify the resistance phenotypes (3).

Results and discussions

Results of the bacteriological examination

During the study 26 strains belonging to the *Staphylococcus* genus were isolated.

Morphology. All 26 Gram-stained bacterial strains were Gram-positive, having the form of cocci with a size of 0.8-1.5 mm in diameter, and the predominant grouping was in bunch, and occasionally in pair, or solitary bacterial cells.

In this study 26 strains of *Staphylococcus* were isolated, of which 19 coagulase-positive strains (CoP, represented by *S. hyicus* and *S. aureus*) and 7 coagulase-negative strains (CoN, represented by *S. haemolyticus*, *S. sciuri* and *S. epidermidis*) isolated from milk from cows suffering from clinical and subclinical mastitis.

Results of the antibiotic sensitivity test

The *Staphylococcus* strains that haven't been exposed to antibiotic pressure are susceptible to these substances, however, the strains isolated from dogs and cats with various conditions under antibiotic pressure due to the therapy may present multiple resistance phenomena.

Analyzing the results it can be observed that the sensitivity to antibiotics was variable depending on the antibiotic groups.

In the case of antibiotics: novobiocin, rifampicin, pristinamycin, ciprofloxacin, vancomycin, ceftriaxone, ceftiofur, cefaclor and ampicillin/sulbactam, being considered *Staphylococcus* elective antibiotics, the percentage of the

susceptible strains was 100%. This suggests that the isolated and tested strains originated from animals where these antibiotics were not used. It can also be said that all these antibiotics constitute the kit for staphylococci, or are usually used in humans in the therapy of infections with *Staphylococcus*, respectively in animals.

To beta-lactamins used (methicillin, ceftriaxone, cefoxitin, cefaclor, ampicillin with sulbactan), the sensitivity to the antibiotics was maximal, except methicillin where 7 resistant strains were isolated. Of these, two strains of *S. hycus* were resistant to methicillin, 4 strains of *S. aureus* and one strain of *S. epidemidis*. The strains tested were largely sensitive to beta-lactamins as a result of previous correctly done treatments.

The antibiotic resistance phenomenon, in the case of beta-lactamins, is based on plasmid and chromosomal genetic determinants that govern the synthesis of beta-lactamins, with a broad spectrum thus providing staphylococci resistance.

The resistance to methicillin is transmitted by plasmids (factor R) having a common pattern over other beta-lactamins. For this reason, methicillin-resistant staphylococci strains are considered strains with a special zoonotic risk, having a complex circuit, namely man-animal-man (1, 5, 7, 8, 10).

To aminoglycosides (gentamicin, kanamycin) and macrolides (erythromycin, and vancomycin) the antibiotic susceptibility was different, being maximal for vancomycin. In this case, different strains have been isolated, 8 strains were resistant to gentamicin, 7 strains showed resistance to kanamycin, 9 to tetracycline, 9 to doxycycline, 11 strains to erythromycin, and 14 to lincomycin.

Most strains showed resistance to polymyxin B (24 strains) due to the use of locally applied preparations containing this antibiotic, in the past.

The antibiotic resistance to tetracycline (tetracycline, doxycycline) was reduced, with 18 strains resistant to this group of antibiotics, where the resistance phenomenon is plasmidic and chromosomal (9 strains of tetracycline and 9 of doxycycline). All strains tested were showed sensitivity to ciprofloxacin because this quinolone is not used in the therapy of diseased bovines.

The development of staphylococcal resistance to various antibiotics is a consequence of the unreasonable use in the therapy of certain diseases in cattle, especially those of the mammary gland. The non-ionic antibiotics used create a selection pressure selecting and transmitting genetic determinants of the plasmid and chromosomal type. Consequently, the phenomenon of multiple resistance occurs which is transmitted in- and interspecific. The resistance to methicillin shows highly importance for it can be associated with the resistance to beta-lactamins and other antibiotic groups (5, 9, 10). After testing the strains isolated from milk, coming from bovine suffering from mastitis, a resistance to 17 antibiotics has been identified, methicillin-resistant strains and several resistotypes, to beta-lactamins, tetracycline, macrolides, polymyxin B. The data on resistance to

methicillin and the identified resistotypes are similar to the results reported by other authors, on the antibiotic resistance phenomenon.

Zhang et al. (11) have investigated, during a study, the strains of *S. aureus* isolated from milk coming from cows that have mastitis, in eastern China. Of the 200 samples of milk analyzed, 58 were positive for *S. aureus*, of which 11 were strains of *S. aureus* (MRSA) methicillin-resistant. Hata (4), in a study conducted in Japan on mastitis outbreaks in cows, produced by *Staphylococcus aureus* methicillin resistant strains (MRSA), out of 78 cows tested, 31 cows were MRSA-bearing, and they were detected by testing the milk coming from lactating cows.

In Egypt, El-Ashker et al. (2), a detailed study has been carried out on the structure of the *Staphylococcus aureus* population in a modern farm of dairy cows (Gamasa) and buffaloes in Dakahlia Governorate, Egypt. A number of 872 milk samples, coming from 218 animals with clinical and subclinical mastitis, have been investigated. *Staphylococcus aureus* has been identified in 5.6% of all collected samples, while the methicillin-resistant strains (MRSA) accounted for 24.5% of all identified *S. aureus* strains (12/49).

Nemeghaire et al. (6) reported a prevalence of MRSA strains in females of 19.8%. All the strains isolated were multidrug resistant, to at least two antimicrobial agents, in addition to cefoxitin and penicillin, with an average resistance to various microbial agents of 9.5. The isolated strains showed a wide range of antibiotic resistance genes, and virulence genes.

Over the past 25 years there has been a worldwide increase in the prevalence of methicillin-resistant *Staphylococcus aureus* strains. Furthermore, in many cases, MRSA infections occurred outside the hospitals, independently, caused by strains of community associated MRSA (CA-MRSA). In Germany, at least 10% of these sporadic infections are due to MRSA strains associated with animal breeding (LA-MRSA).

Conclusions

It has been confirmed that staphylococci are an important cause in the occurrence of mastitis in buffaloes, and the existence of methicillin-resistant strains, and the multi-resistance phenomenon have been demonstrated.

The antibiotic susceptibility of strains isolated from milk coming from diseased animals, was variable, depending on the antibiotic groups:

- to beta-lactams used (methicillin, ceftriaxone, cefoxitin, cefaclor, ampicillin with sulbactam), the antibiotic susceptibility was maximal, except to methicillin, as resistant strains were isolated;
- as regards aminoglycosides (gentamicin, kanamycin) and macrolides (erythromycin and vancomycin), the antibiotic susceptibility was different, being maximal for vancomycin. A phenomena of resistance have been described for gentamicin, kanamycin, erythromycin and lincomycin, respectively;

- the antibiotic susceptibility to tetracycline (tetracycline, doxycycline) has been increased;
- all the isolated strains were resistant to polymyxin B and sensitive to ciprofloxacin.

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MEGAESOPHAGUS DUE TO MYASTHENIA GRAVIS IN DOG – CASE REPORT

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Summary

A 9 years old male mixed breed of shepherd dog was diagnosed with megaesophagus. The subject showed evident clinical signs of a severe pathological state, occurred in about two weeks. The symptoms were cough, dyspnea, dysphagia, regurgitation, general weakness state and fever. Megaesophagus was confirmed by radiography, which revealed the dilation of the thoracic part of the organ. It was not possible to do the specific test for the Myasthenia gravis but the specific clinical signs lead to the diagnostic. The co-existence of Myasthenia gravis and megaesophagus usually occurs in small animals, in particular in dogs, and it starts up with megaesophagus as an initial symptom of Myasthenia gravis. The disease management was difficult because of the complication with aspiration pneumonia, which caused the death of the patient due to cardiorespiratory insufficiency.

Keywords: dog, megaesophagus, Myasthenia gravis, aspiration pneumonia

Background

Acquired myasthenia gravis (MG) in dogs is an autoimmune disease affecting the neuromuscular junction. Muscular weakness and excessive fatigability result from autoantibody mediated destruction of nicotinic AChRs of the neuromuscular junction (5). Megaesophagus is often associated to myasthenia gravis. The clinical signs of megaesophagus are based on the neural pathways disturbance, though the pathogenesis is not very clear (9, 14).

Case presentation

The dog was referred to the clinic due to vomiting and profuse regurgitation few minutes after eating, and also for unusual fatigue after exercise. The symptoms started up several days before the visit to the veterinarian.

The dog was a mixed breed of Shepherd, 9 years old, male, 30 kg, with no other diseases in background, except an accidentally poisoning (rodenticide) two years before, without any long term consequences. The dog was normally fed with dry food and it was an indoor and outdoor too.

Clinical examination revealed cough, dyspnea, dysphagia, fever and general weakness state (the dog could not stand up). Blood tests were performed by using IDEXX integrated system. The results were not very critic despite the

severe condition. However, an increase in leukocytes value, in particular in segmented neutrophils was noticed (Fig. 2).

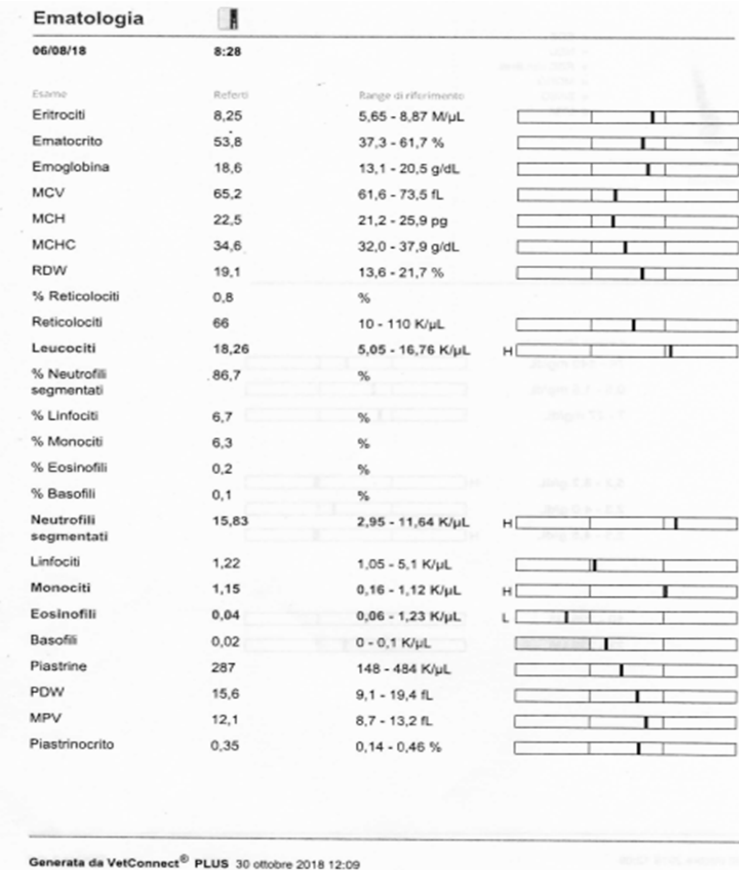


Fig. 2. Blood test results

In order to reveal morphological abnormalities, direct X-ray examination of the cervical and thoracic segments of esophagus, in lateral and dorso-ventral projection was performed. A moderately dilated cervical esophagus, dorsally to the air column of the trachea, was highlighted on lateral view (Fig. 3). The dilation was considerably higher in the thoracic tract (Fig. 4) up to the diaphragm. It was also noticed the appearance of the gastric bulla less stretched by the air, as another sign of the digestive tract disorder.

Following dorso-ventral projection X-ray (Fig. 5, 6), aspiration pneumonia was diagnosed at the level of the median right lung lobe, based on the well-defined boundaries of the lobe and the visible sign of the "air bronchogram" (pathognomonic for pneumonia).

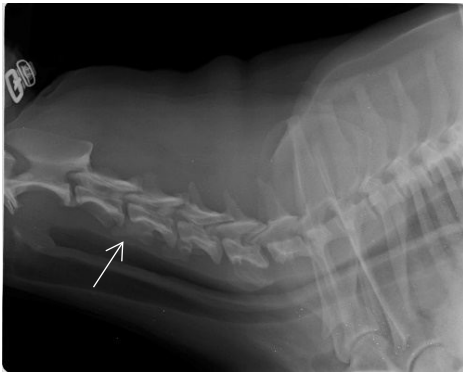


Fig. 3. Dog cervical radiography, lateral projection. Mild esophageal dilation (arrow)

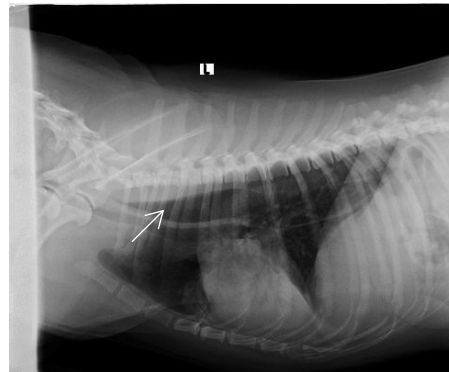


Fig. 4. Dog thoracic radiography, lateral projection. Esophageal dilation (arrow)

Following the X-ray findings megaesophagus was diagnosed. Because the patient was critically debilitated and the general condition worsened rapidly, percutaneous endoscopic gastrostomy (PEG) was performed. 24 hours following surgery it was possible to nourish the dog through the feeding tube and to administer oral drugs.



Fig. 5. Dog thoracic radiography, "soft rays", dorso-ventral projection. Aspiration bronchopneumonia



Fig. 6. Dog thoracic radiography, "hard rays", dorso-ventral projection. Aspiration bronchopneumonia

For the treatment protocol there were considered anticholinesterase, antibiotics and gastroprotective drugs, targeting Miastenia gravis, pneumonia and gastroprotection. Electrolyte fluids were also administered to balance the blood composition. Thus, treatment consisted in administration of Mestinon 1.5 mg/kg b.i.d., Ceftriaxone 30 mg/Kg b.i.d., Metronidazole 10 mg/Kg b.i.d., Ranitidine 2 mg/Kg t.i.d., Omeprazole 1mg/Kg s.i.d., Sucrafate 40 mg/Kg t.i.d. and fluids, according to the electrolytic control tests.

The surgery was a success and the dog retained the food, i.e. was a clear and positive sign that the rest of the digestive tract was preserved. The regurgitation episodes were also diminished and after the Mestinon administration the dog was able to better stand up (thus it was a major proof of the Myasthenia gravis condition).

Unfortunately, because of the severity and aggressiveness of the pathological condition, the patient died following cardio-respiratory failure.

Discussion

Megaesophagus defines a syndrome of segmental or diffuse dilatation of the esophagus due to hypomotility and reduction of normal peristaltic movement or due to obstruction (4). It is known that motility disturbances of esophagus are generated by abnormalities in neural, neuromuscular junction and or muscular dysfunction (3).

Megaesophagus may be congenital or acquired. Congenital megaesophagus is usually idiopathic and was documented in many breeds including the wire-haired Fox-terrier, miniature Schnauzer, Great Dane, Newfoundland, Rhodesian ridgeback and the Chinese Shar-Pei. Clinical signs in congenital megaesophagus most often start shortly after weaning (14). Acquired form of megaesophagus may be primary (idiopathic), or secondary to other diseases such as myasthenia gravis, hypoadrenocorticism, canine dysautonomia, tetanus, polyradiculoneuritis, tick paralysis, lead toxicity, systemic lupus erythematosus, polymyositis and possibly hypothyroidism (8, 14).

In Myasthenia gravis, the receptors to which acetylcholine is bound are blocked by antibodies produced "accidentally" by the immune system (auto-antibodies) and the muscles do not receive the stimuli that regulate their function, thus they are weak and easily tiring. Also, the production of anti-MuSK anti-anti-tyrosine kinase muscle antibodies is documented as a cause for Myasthenia gravis (10, 13, 17). Another possible, but less frequent, cause of Myasthenia is the hereditary disorder resulting in deficiency of Ach receptors on the postsynaptic membrane, that consist in the mutation of CHNRP1 genes, creating defective acetylcholine receptors in fetal or adult muscles (2).

The acquired form of Myasthenia rarely affects animals younger than 1 year of age, and there seem to be two peaks of age in which the disease is more frequent: 2/3 years and 9/10 years. In this form most of the symptoms are: megaesophagus, drooping of one or both eyelids (ptosis), double vision (diplopia) change in facial expression, difficult swallowing, shortness of breath, weakness in

the extremities (15). In the congenital form the onset of clinical signs is usually apparent at 6-8 weeks of age, with signs of muscular weakness without presence of megaesophagus (12).

The megaesophagus warning sign is regurgitation. It is often present in puppies while in older patients is variable. (6). A common complication of megaesophagus is aspiration pneumonia, manifested by respiratory signs (cough, dyspnea) (4). Other signs are: weight loss (cachexia), vomiting, nasal discharge, anorexia, ptyalism and halitosis (16). Food stasis into the esophagus causes fermentation and esophagitis (7).

The symptoms usually start when patients are middle age to older (7-15 years) (8, 14). Prognosis is poor, especially when secondary (aspiration) pneumonia is present (3).

Idiopathic megaesophagus is managed with supportive and symptomatic treatment, with periodic rechecks. The treatment for the acquired form depends on the primary disease management, in addition to the supportive and symptomatic care. Esophageal dilation and aspiration pneumonia are monitored through radiography (6).

In order to reduce the symptoms, cholinesterase inhibitors, corticosteroids and immunosuppressants are administered. Cholinesterase inhibitors indirectly increase the bioavailability of acetylcholine and so that it can compete with autoantibodies for binding to its receptors on the neuromuscular junction (1). Corticosteroids interfere with the immune system's response, and immunosuppressants can suppress the immune system. Thus, the autoimmune response which is triggered against neuromuscular junction's cholinergic receptors can be decreased (15).

Also, if the animal is not able to eat, it is important to manage the pathological condition by forced feeding, directly into the stomach through a feeding tube (16). If the patient is able to eat, the feeding should be frequent, with small, high-calorie meals with the patient in a cranially elevated position. This position uses gravity to help the ingesta to move to the stomach (14). An upright position for 10 to 15 minutes after eating or drinking is recommended (16).

The case was presented because of its acuity and criticality. It developed in only two weeks within which all the symptoms showed up. Before the onset, the only factor reported by the owner, two-three weeks before the outbreak of the disease, was a major fatigue after exercise (e.g. after the usual walk), the dog appeared more tired and breathless. These symptoms were not properly taken in consideration by the owner because the dog, as the owner described, was physically healthy and strong, never diseased before. Moreover, when the pathology manifested, the environmental temperature was high because of the summer season; thus, the owner imputed the fatigue to the high temperature.

There was no really evidence about the correlation between the fatigue and what happened afterwards but in any case is advisable for owners to pay attention to any behavioral change of their dogs and for veterinarians to get every detail by a good anamnesis.

The case also shows that, except other causes, every time when there is a situation such as: prolonged and profuse regurgitation/vomiting, a German shepherd breed or a mixed breed of shepherd, a range of age at risk (7-15 years old) and progressive asthenia to always take into account the possibility of megaesophagus.

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**COMPARATIVE RESEARCH ON ANTIMICROBIAL RESISTANCE
IN BACTERIA ISOLATED FROM DOMESTIC AND WILD ANIMALS
(CHAMOIS - *RUPICAPRA RUPICAPRA*)**

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Summary

Monitoring the circulating pathogens in domestic and wildlife populations is extremely important in order to understand the transmission and evolution of bacterial diseases between wild and domestic animals as well as the epidemiological circuit between them.

The research aimed the frequency of resistance phenotypes in eight bacterial strains, isolated and identified from wild living chamois (*Rupicapra rupicapra*) from alpine environments, compared to strains isolated from domestic animals (dogs). The eight strains from the chamois isolated from faeces were included in *L. monocytogenes*, *E. coli* and *Salmonella spp.*, based on cultural and morphological characters, as well as by the use of selective chromogenic media.

Antimicrobial resistance was determined by the Kirby-Bauer disc-diffusion method using, for this purpose, Mueller-Hinton medium and biodiscs with nine antibiotics. The results showed a higher resistance to strains collected from domestic animals compared to wild animals, with higher resistance to colistin sulphate and penicillin G, followed by tetracycline, amoxicillin with clavulanic acid and streptomycin.

Keywords: antimicrobial resistance, bacteria, chamois, phenotypes

Since the beginning of 1950, the phenomenon of antibiotic resistance in bacteria has been reported, especially after the introduction of sulfonamides and antibiotics in the prophylaxis and therapy of some infectious diseases in animals and humans. The expansion of antibiotic resistance phenomenon in Gram positive and Gram negative bacteria, pathogenic both to animals and humans, required extensive studies conducted over time (1, 2).

Thus, monitoring the circulating pathogen agents both in domestic and, as well, in wildlife populations represents an extremely important issue in order to understand the transmission and evolution of bacterial diseases between wild and domestic animals, the epidemiological circuit between them and as well between them and humans (1, 2).

The research was made in order to highlight the presence of resistance phenotypes in bacterial strains isolated from wild animals and to compare them with isolates from domestic animals.

Materials and methods

The researches aimed the frequency of resistance phenotypes in 8 bacterial strains, isolated and identified from wild living chamois (*Rupicapra rupicapra*) from alpine environments, the mountain area of Retezat, Romania. These 8 strains from chamois were isolated from the faeces (minimum 15 g for each sample).

Primary inseminations (approximately 4 g for each sample) were performed in nutrient broth, and the incubation, in aerobiose, lasted 24 hours at 37°C. Subsequent inseminations were then made on the nutrient agar and the isolated strains were sorted based on cultural, morphological and tinctorial characters. All strains were inseminated on chromogenic and selective media (Levine, Oxford and Rambach) and based on it, included in *L. monocytogenes*, *E. coli* and *Salmonella spp.*

Antibiotic resistance patterns were determined by Kirby-Bauer disc-diffusion method, using for this purpose the Mueller-Hinton medium and biodiscs with nine antibiotics: amoxicillin with clavulanic acid (AMC), colistin sulphate (CT), florfenicol (FFC), neomycin (N), nitrofurantoin (F), norfloxacin (NOR), penicillin G (P), streptomycin (S) and tetracycline (TE).

The results were interpreted according to the recommendations of the Institute for Clinical Laboratory Standards, USA (CLSI-Clinical and Laboratory Standard Institute) taken by the European Committee for Testing the Antibiotic Susceptibility of the European Society of Infectious Diseases and Clinical Microbiology and the strains were classified into three categories: susceptible, intermediate and resistant.

The obtained results were compared with isolates from domestic animals (dogs), strains which were subjected to the same tests for antimicrobial resistance.

Results and discussions

For the rapid discrimination of bacterial strains isolated from chamois, three chromogenic and selective media were used, on the basis of which several bacterial species could be identified. Thus, based on of this phenotypic character, the isolated strains were included into the following species: *L. monocytogenes* (4), *E. coli* (2) and *Salmonella spp.* (2).

The strains isolated from chamois came from healthy animals that live freely in the alpine environments. They presented several patterns of resistance, which are shown in Table 1. These resistance phenotypes were compared with those from domestic animals, respectively dogs, shown in Table 2. For the detection of resistance patterns, nine antibiotics were used, included in several classes, some of which were also associated with beta-lactamase inhibitors. Using the Kirby-Bauer disc-diffusion phenotypic method, several resistance phenotypes were identified, the number and frequency of which varied according to the species of animal from which the strains originated (Fig. 1).

Table 1

Antibiogram results of strains isolated from chamioses

Crt. No.	Antibiotic	Antibiogram results						Total strains
		S		I		R		
		No.	%	No.	%	No.	%	
1.	Amoxicillin/ clavulanic acid	0	0	3	37.5	5	62.5	8
2.	Colistin sulphate	0	0	0	0	8	100	8
3.	Florfenicol	8	100	0	0	0	0	8
4.	Neomycin	5	62.5	3	37.5	0	0	8
5.	Nitrofurantoin	7	87.5	1	12.5	0	0	8
6.	Norfloxacin	4	50	1	12.5	3	37.5	8
7.	Penicilină G	0	0	0	0	8	100	8
8.	Streptomycin	2	25	2	25	4	50	8
9.	Tetracycline	0	0	1	12.5	7	87.5	8

Table 2

Antibiogram results of strains isolated from dogs

Crt. No.	Antibiotic	Antibiogram results						Total strains
		S		I		R		
		No.	%	No.	%	No.	%	
1.	Amoxicillin/ clavulanic acid	0	0	1	12.5	7	87.5	8
2.	Colistin sulphate	0	0	0	0	8	100	8
3.	Florfenicol	8	100	0	0	0	0	8
4.	Neomycin	1	12.5	3	37.5	4	50	8
5.	Nitrofurantoin	3	37.5	3	37.5	2	25	8
6.	Norfloxacin	1	12.5	4	50	3	37.5	8
7.	Penicilină G	0	0	0	0	8	100	8
8.	Streptomycin	1	12.5	2	25	5	62.5	8
9.	Tetracycline	0	0	0	0	8	100	8

Analyzing the results, it is noted that the resistance patterns had a variable frequency depending on the antibiotic group against which the test was performed.

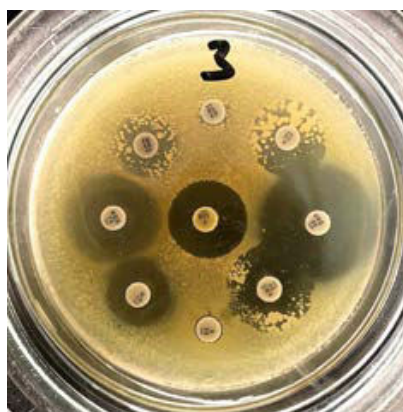


Fig. 1. Strain isolated from chamois with multiple resistance

The frequency of resistance patterns at the strains isolated from chamois was minimal (37.5%) to NOR and maximum (100%) to CT and P. Compared to these, the resistance patterns at the strains isolated from dogs had a minimum resistance (25%) to F and maximum (100%) to 3 antibiotics, namely CT, P and TE. The frequency of strains with intermediate resistance at the isolates from chamois ranged from 12.5% (F, NOR and TE) and 37.5% (AMC and N), while the susceptible strains had a frequency between 25% (S) and 100% (FFC). On the other hand, the strains isolated from dogs, had an intermediate resistance ranging between 12.5% (AMC) and 50% (NOR) and the sensitivity between 12.5% (N, NOR and S) and 100% (FFC).

Beta-lactams resistance patterns were followed using two antibiotics, one of which was also associated with beta-lactamase inhibitors. The resistance patterns at the isolates from chamois had a frequency of 62.5% (AMC), respectively 100% (P), compared to the patterns of resistance at the strains isolated from dogs, the frequency of which was 87.5% (AMC) and 100% (P). Intermediate strains had a frequency of 37.5% (AMC) at isolated strains from chamois and 12.5% (AMC) at the strains isolated from dogs. There were no susceptible strains to penicillin G, both in strains isolated from chamois and also from dogs. Analyzing the behavior of the strains tested against beta-lactam it is noticed that the frequency of strains isolated from the chamois was approximately equal to the frequency of resistant strains isolated from dogs, with no susceptible strains to any of the isolated strains.

From the **aminoglycoside** group, resistance phenotypes were made for two antibiotics, namely neomycin and streptomycin. Thus, the resistance patterns at the strains isolated from chamois had a frequency of 50% (S), while the resistance patterns at the strains isolated from dogs had a frequency of between 50% (N) and 62.5 % (S). Intermediate behavioral strains had a frequency ranging

from 25% (S) to 37.5 (N) in both of the strains isolated from chamois and dogs. The strains susceptible to neomycin had a frequency of 62.5% at the strains isolated from chamois, unlike the strains isolated from dogs, where the frequency was 12.5%. The strains susceptible to streptomycin isolated from chamois had a frequency of 25% and the strains isolated from dogs had a frequency of 12.5%. Analyzing the behavior of these strains towards aminoglycosides, it appears that the frequency of resistance patterns at the strains isolated from dogs was much higher in both tested antibiotics than the strains isolated from the chamois which did not show any resistance pattern to neomycin.

The frequency of antibiotic resistance patterns from the **tetracycline** group was established using only the tetracycline alone. The isolated strains from the chamois had a frequency of 87.5% compared to the isolated strains from the dogs, whose frequency was 100%, whereas there were no susceptible strains to this antibiotic at neither of the tested strains.

For the determination of resistance patterns to **fluoroquinolones**, only norfloxacin was used. The resistant strains isolated from both chamois and dogs had the same frequency of 37.5%, while the susceptible strains isolated from chamois had a frequency of 50%, unlike the susceptible strains isolated from dogs, which had frequency only of 12.5%. Intermediate behavioral strains had a higher frequency at the strains isolated from dogs (50%) than the strains isolated from chamois (12.5%).

The frequency of resistance patterns to **nitrofurans** was established, only for nitrofurantoin. Thus, the frequency of resistant strains isolated from dogs was 25%, while the isolates from chamois had no resistance patterns. The frequency of susceptible strains isolated from chamois was high, of 87.5%, compared with the strains isolated from dogs, which had a sensitivity frequency of 37.5%.

Of the **polypeptide** group, the resistance of the strains was tested only for colistin sulphate. The frequency of resistant strains isolated from both the chamois, and the dogs was 100%, thus there weren't any susceptible strains or strains with intermediate behavior to this antibiotic.

To **florfenicol** there were no resistance patterns to any of the tested strains, regardless of species and the susceptible strains had a frequency of 100% in the strains isolated from both chamois and dogs.

The frequency of resistance phenotypes at the strains isolated from both domestic and wild animals represent an important area of infectious pathology, which is why numerous collective study this epidemiological issue in many countries. The results obtained showed the presence of the antibiotic resistance phenomenon at the strains isolated from wild animals, respectively the chamois, phenomenon that was also noted by other researchers.

Thus, in 2012 in Switzerland, Stephan and Häcler studied the antibiotic resistance phenomenon and the identification of β -lactamase producing *E. coli* strains in wild animals. For this purpose, faecal samples were taken from 84 red deer, 64 roe deer, 64 chamois and 27 ibex. From these samples, a single ESBL

producing *E. coli* strain was isolated, tested on chromogenic media and definitively identified by API ID 32 E system. This strain was subjected to the antibiogram, the results being the following: it was resistant to ampicillin, amoxicillin with clavulanic acid, cephalothin, cefuroxime, cefpodoxime, cefotaxime, ceftazidime, cefepime and tetracycline and susceptible to ceftiofur, imipenem, ciprofloxacin, nalidixic acid, gentamycin, streptomycin, polymyxin B and trimethoprim-sulfamethoxazole (4).

Also in 2012, in Slovakia, Vandžurová A. et al. studied the phenomenon of antimicrobial resistance and the presence of restriction endonucleases in faecal enterococci strains from chamois. The authors isolated 284 strains of faecal enterococci that were tested against three antibiotics, respectively ampicillin, erythromycin and tetracycline. They identified a small frequency of resistant enterococci phenotypes and only for two of the three tested antibiotics (5%) resistant phenotypes to erythromycin and tetracycline, compared to ampicillin which had no resistance phenotypes (5).

In Italy, in 2014, Luzzago C. et al. have studied the antimicrobial resistance profile as well as virulence-associated genes in *Staphylococcus aureus* strains isolated from nasal cavities and soft tissue infections from wild ruminants. The samples were taken from two chamois and one roe deer, and the isolates of staphylococci were tested for the antimicrobial resistance to penicillin G, ampicillin, amoxicillin with clavulanic acid, ceftiofur, ceftriaxone, enrofloxacin, ciprofloxacin, tetracycline, doxycycline, trimethoprim-sulfamethoxazole, erythromycin, streptomycin, kanamycin, gentamicin and tobramycin. Only one chamois isolate showed resistance to penicillin, ampicillin, amoxicillin with clavulanic acid, ceftiofur, ceftriaxone, ciprofloxacin and enrofloxacin, and the methicillin resistance was confirmed by the presence of the *mecA* gene, performed by PCR techniques (3).

The results obtained in this study are similar to the results obtained by the cited groups of researches, thus demonstrating the presence of resistance patterns in wild animals against some antibiotics used in therapy, probably due to the epidemiological circuit of bacterial strains between wild and domestic animals.

Conclusions

A number of eight strains were isolated from the chamois and, based on the cultural, morphological and chromogenic media used, were included in three species.

The disc-diffusion method revealed a higher resistance to the isolates from domestic animals, respectively the dogs, compared to the isolates from wild animals, respectively the chamois.

Both in strains isolated from wild animals, as well as in the ones isolated from domestic animals, the frequency of resistant strains was highest to colistin sulphate and penicillin G, followed by tetracycline, amoxicillin with clavulanic acid and streptomycin.

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PREVALENCE OF CANINE GASTROINTESTINAL HELMINTHS IN TIMIȘOARA

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Summary

In Romania, the number of dogs with owners, as well as stray dogs, has increased significantly in recent years. The level of environmental pollution with parasitic elements has also increased, some of them of great importance to humans.

In this study, 207 dogs (103 strays from the public shelter and 104 dogs with owners) from Timișoara were investigated for gastrointestinal helminths, using the Willis flotation method. The prevalence of nematodes in fecal samples gathered from owned dogs, in descending order, was: *Trichocephalus vulpis* (42.85%), *Toxocara canis* (36.73%), *Ancylostoma caninum* (14.28%), *Toxascaris leonina* (4.08%) and *Strongyloides stercoralis* (0.96%). In shelter dogs, the following prevalence was recorded: *Ancylostoma caninum* (97%), *Toxascaris leonina* (32%) and *Toxocara canis* (19%). The occurrence of parasitism was found to be high among the age group of 0-6 months and in large breeds and mixed breeds. The most common monoparasitism was with *Ancylostoma caninum* (25%), found in the public shelter. In the case of multiparasitism, the most frequent combination was *Ancylostoma caninum* and *Toxascaris leonina* (11.5%).

Keywords: gastrointestinal helminths, prevalence, public shelter

Dogs offer substantial benefits such as emotional development, socialization and physiological well-being, but they can also be reservoirs for a large number of parasitic zoonoses, such as: toxoplasmosis, giardiasis, toxocariasis and ancilostomiasis (1, 2, 4). This is important because the parasitic elements can penetrate the human body through different pathways, the most commonly involved being cutaneous, digestive and respiratory (4, 8, 11). Man can host over 100 different types of parasites. It is estimated that 85% of the adult population have at least one form of parasite living in their body (12, 20).

Materials and methods

The aim of the study was to identify the prevalence of gastrointestinal parasites in dogs from the public shelter and dogs with owners, from Timișoara, in accordance with their age and breed and by evaluating the presence of monoparasitism and multiparasitism.

In this study, 207 dogs (103 community dogs from the public shelter and 104 dogs with owners) from Timișoara were examined. The dogs belonged to

different age categories and those with owners were divided into: 0-6 months (33/104), 7-12 months (14/104), 1-3 years (35/104) and > 3 years (22 / 104). The dogs with owners included in the study belonged to various breeds: Akita Innu (1/104), American Pitt Bull (1/104), Amstaff (6/104), Bichon (17/104), German Short-haired Pointing Dog (2/104), French Bulldog (4/104), Bull Terrier (2/104), Cane Corso (2/104), Chihuahua (1/104), Chow Chow (2/104), Carpathian Shepherd (1/104), Central Asian Shepherd (1/104), Bucovina Shepherd (1/104), German Shepherd (5/104), Labrador Retriever (4/104), Malinois Shepherd (2/104), Mixed breeds (34/104), Pekingese (1/104), Poodle (1/104), Presa Canario (1/104), Rottweiler (2/104), Giant Schnautzer (2/104), Shar Pei (1/104), Shih Tzu (1/104), Teckel (3/104), West Highland White Terrier (2/104), Vizsla (1/104) and Yorkshire Terrier (4/104).

The stool samples were collected individually by the owner of the animal or by the public shelter employees. For each sample a qualitative coproscopic examination was performed with the Willis flotation method and the direct method with Lugol solution, according to the protocol described by Cosoroabă et al. (2002) (4). In dogs with owners, a rapid test for the detection of *Giardia* spp. was performed. The parasitic eggs have been identified according to their morphological characteristics, as described by Mehlhorn et al. and Cosoroabă et al. and the percentages for each pathogen were calculated (4, 16).

In comparison, in public shelters and veterinary clinics, the evolution and importance of monoparasitism and multiparasitism has also been evaluated.

Results and discussions

Several nematodes and also protozoans and tapeworms, such as: *Isoospora canis*, *Giardia* spp., *Taenia* spp., *Dipylidium caninum*, *Toxocara canis*, *Toxascaris leonina*, *Ancylostoma caninum*, *Trichocephalus vulpis* and *Strongyloides stercoralis* were discovered in the 104 fecal samples collected from dogs with owners (Table 1). The lowest prevalence was 0.96% (1/104) for *Strongyloides stercoralis* and the highest was 20.19% (21/104) for *Trichocephalus vulpis*, followed by *Toxocara canis* with a prevalence of 17.30% (18/104).

Regarding the prevalence of the identified nematodes, the predominance of parasitism with *Trichocephalus vulpis*, followed by *Toxocara canis*, *Ancylostoma caninum* and *Toxascaris leonina*, was observed (Table 2).

The prevalence of gastrointestinal parasites in dogs with owners was also expressed according to age. The dogs were divided into four age groups: 0-6 months, 7-12 months, 1-3 years, > 3 years (Table 3).

The results obtained in the studied dog breeds revealed a more intense parasitism in large breeds with access to the yard and implicit contact with soil parasites. The highest number of infested dogs was observed in mixed breeds (35%), followed by large breeds such as German Shepherd (14%), Amstaff (9%), Labrador Retriever (5%), Bull Terrier (4%) and small breeds such as Bichon (14%),

Yorkshire Terrier (5%), French Bulldog (5%) and Dachshund Dog (4%). No internal parasites were found in breeds like Akita Inu, Central Asian Shepherd, Bucovina Shepherd, Malinois Shepherd, Pekingese, Poodle, Shih Tzu, West Highland White Terrier and Hungarian Vizsla.

Table 1

The prevalence of parasitism in dogs with owner

No.	Type of parasites identified	Prevalence (%)
1	<i>Isospora canis</i>	9/104 (8.65%)
2	<i>Giardia spp.</i>	3/104 (2.88%)
3	<i>Taenia spp.</i>	6/104 (5.76%)
4	<i>Dipylidium caninum</i>	2/104 (1.92%)
5	<i>Toxocara canis</i>	18/104 (17.30%)
6	<i>Toxascaris leonina</i>	2/104 (1.92%)
7	<i>Ancylostoma caninum</i>	7/104 (6.73%)
8	<i>Trichocephalus vulpis</i>	21/104 (20.19%)
9	<i>Strongyloides stercoralis</i>	1/104 (0.96%)

Table 2

The prevalence of gastrointestinal nematode parasitism in dogs with owner

No.	Type of parasites identified	Prevalence (%)
1	<i>Toxocara canis</i>	18/49 (36,73%)
2	<i>Toxascaris leonina</i>	2/49 (4,08%)
3	<i>Ancylostoma caninum</i>	7/49 (14,28%)
4	<i>Trichocephalus vulpis</i>	21/49 (42,85%)
5	<i>Strongyloides stercoralis</i>	1/49 (2,04%)

Table 3

The prevalence of gastrointestinal parasites in dogs with owners according to age

No.	Age	Positive/Total (%)	Gastrointestinal parasites identified
1	0-6 months	21/33 (63.63%)	<i>Giardia spp.</i> , <i>Isospora canis</i> , <i>Dipylidium caninum</i> , <i>Toxascaris leonina</i> , <i>Toxocara canis</i> , <i>Trichocephalus vulpis</i> , <i>Ancylostoma caninum</i>
2	7-12 months	8/14 (57.14%)	<i>Giardia spp.</i> , <i>Isospora canis</i> , <i>Dipylidium caninum</i> , <i>Taenia spp.</i> , <i>Strongyloides stercoralis</i> , <i>Toxocara canis</i>
3	1-3 years	22/35 (62.85%)	<i>Taenia spp.</i> , <i>Toxocara canis</i> , <i>Trichocephalus vulpis</i> , <i>Ancylostoma caninum</i>
4	>3 years	11/22 (50%)	<i>Taenia spp.</i> , <i>Toxascaris leonina</i> , <i>Toxocara canis</i> , <i>Trichocephalus vulpis</i> , <i>Ancylostoma caninum</i>

Ancylostoma caninum (97.08%), followed by *Toxascaris leonina* (32.03%) and *Toxocara canis* (19.41%) were identified in the 103 samples of feces obtained from public shelter dogs (Table 4).

Table 4

The prevalence of parasitism in dogs from the public shelter

No.	Type of parasites identified	Prevalence (%)
1	<i>Ancylostoma caninum</i>	100/103 (97.08%)
2	<i>Toxocara canis</i>	20/103 (19.41%)
3	<i>Toxascaris leonina</i>	33/103 (32.03%)
4	<i>Trichocephalus vulpis</i>	14/103 (13.59%)

In the 207 samples investigated, both monoparasitism and multiparasitism were present. The most frequent monoparasitism was the one with *Ancylostoma caninum* nematode (52/207), occurring in 25% of cases. This type of parasitism has been identified especially in dogs from the public shelter. Second place was the monoparasitism with 10.14% *Trichocephalus vulpis* (21/207), followed by *Toxocara canis* with 7.72% (16/207) (Table 5). As for multiparasitism, the most frequent association was between *Ancylostoma caninum* and *Toxascaris leonina* in 24 cases (11.59%) followed by *Ancylostoma caninum* and *Toxocara canis* in 12 cases (5.79%) and *Ancylostoma caninum* and *Trichocephalus vulpis* in 9 cases (4.34%) (Table 6).

Table 5

The prevalence of monoparasitism in dogs

No.	Monoparasitism	Positive/ Total (%)
1	<i>Isospora canis</i>	7/207 (3.38%)
2	<i>Giardia</i>	2/207 (0.96%)
3	<i>Taenia spp.</i>	5/207 (2.41%)
4	<i>Dipylidium caninum</i>	2/207 (0.96%)
5	<i>Toxocara canis</i>	16/207 (7.72%)
6	<i>Toxascaris leonina</i>	2/207 (0.96%)
7	<i>Ancylostoma caninum</i>	52/207 (25.12%)
8	<i>Trichocephalus vulpis</i>	21/207 (10.14%)
9	<i>Strongyloides stercoralis</i>	1/207 (0.48%)

Table 6

The prevalence of multiparasitism in dogs

No.	Multiparasitism	Positive/ Total (%)
1	<i>Isospora canis</i> <i>Taenia spp.</i>	1/207 (0.48%)
2	<i>Isospora canis</i> <i>Toxocara canis</i>	1/207 (0.48%)
3	<i>Giardia</i> <i>Toxocara canis</i>	1/207(0.48%)
4	<i>Toxocara canis</i> <i>Ancylostoma caninum</i>	12/207 (5.79%)
5	<i>Toxascaris leonina</i> <i>Ancylostoma caninum</i>	24/207 (11.59%)
6	<i>Ancylostoma caninum</i> <i>Trichocephalus vulpis</i>	9/207 (4.34%)
7	<i>Ancylostoma caninum</i> <i>Trichocephalus vulpis</i> <i>Toxocara canis</i>	1/207 (0.48%)
8	<i>Ancylostoma caninum</i> <i>Trichocephalus vulpis</i> <i>Toxascaris leonina</i>	2/207 (0.96%)
9	<i>Ancylostoma caninum</i> <i>Toxocara canis</i> <i>Toxascaris leonina</i>	4/207 (1.93%)
10	<i>Ancylostoma caninum</i> <i>Toxocara canis</i> <i>Toxascaris leonina</i> <i>Trichocephalus vulpis</i>	3/207 (1.44%)

In a similar study carried out in Warsaw, Poland, by *Borecka*, in 2005, the prevalence of gastrointestinal nematode infection was evaluated in 2659 dogs from public shelters, rural areas and boarding houses. Eggs of *Toxascaris leonina*, *Toxocara canis*, *Trichuris vulpis* and *Ancylostomatidae* have been identified. In concordance with the results of the current study, the highest prevalence was recorded in public shelters, respectively 71.2% in the first shelter, 56.5% in the second shelter and 80.9% in third shelter, a lower prevalence than those recorded in Timis county shelter (97%). Also, a lower prevalence can be seen in the second shelter compared to the first and third shelter, the authors believing that the lower-prevalent shelter had superior hygienic conditions compared to the other two. Also, a higher prevalence (34.2%) was recorded in rural dogs compared to dogs from Warsaw city (3.3%). The study affirms that the external environment has an important influence on dog parasitism (2).

The results of the presented studies showed an infestation with *Toxascaris leonina*, especially in city free-ranging dogs, with a prevalence of 32%. *Ancylostoma caninum* was the most common parasite identified in community dogs from the public shelter. In dogs with owners, *Toxascaris leonina* was identified just in one sample compared to the study conducted in the three public shelters, in Poland, where this parasite was diagnosed in 14.2% of the dogs from the first shelter, 0.3% in the second shelter and 15.8% in the third shelter. Other authors identified this nematode in 0.9% of dogs with owners, in Poznafi (Western Poland), 1%, in Olsztyn (North Poland) and 5%, in Wroclaw (Southern Poland) (8, 15, 18).

Whipworm infestation was found in the present study, in large percentage, in dogs with owners (20.1%) but also, in dogs from shelters (13.59%). *Okulewicz et al.* (1994) identified *Trichocephalus vulpis* infestations in about 40% of the dogs in the Wroclaw region, Poland. This nematode has been detected in free-ranging dogs in all European countries, in Serbia (18.1%), Belgium (7%), Italy (10%), Spain (8.2%) (12). The prevalence of *T. vulpis* infestation was lower in Czech Republic (0.9%) and in Netherlands (0.7%) (21, 25).

In Greece, in a study carried out by *Papazahariadou et al.*, in 2007, in Prefecture Serres, Northern Greece, 281 samples of feces were collected from shepherd dogs and hunting dogs and examined for the presence of intestinal parasites. The prevalence of parasitism was 26% and the 11 parasitic species found were *Toxocara canis* (12.8%), *Trichocephalus vulpis* (9.6%), *Giardia spp.* (4.3%), *Isospora (Cystoisospora) spp.* (3.9%), *Ancylostoma / Uncinaria spp.* (2.8%), *Cryptosporidium spp.* (2.8%), *Alaria alata* (2.5%), *Strongyloides stercoralis* (1.8%), *Angiostrongylus vasorum* (1.1%), *Toxascaris leonina* (0.7%) and *Dipylidium caninum* (0.3%). The prevalence of *T. canis* and *Isospora (Cystoisospora) spp.* was significantly higher in younger dogs than in adult dogs. There was no significant difference in gender prevalence, except for *T. canis*, which was more common in male dogs (22).

In another region in Greece, Crete, *Kostopoulou et al.* conducted a study, in 2017, in which 879 feces samples were collected from dogs with owners,

community dogs from the public shelter and shepherd dogs. Overall, the prevalence was 25.2% for *Giardia spp.*, 9.2% for *Ancylostoma / Uncinaria spp.*, 7.6% for *Toxocara spp.*, 5.9% for *Cryptosporidium spp.*, 4.6% for *Cystoisospora spp.*, 2.7% for *Toxascaris leonina*, 1.7% for *Capillaria spp.*, 0.8% for *Tenia spp.*, 0.2% for *Dipylidium caninum* and 0.1% for *Strongyloides stercoralis*. In this study, more than half of the shepherd's dogs (51.4%) were positive for at least one intestinal parasite species. It is believed that animals, farm dogs and shepherds, often receive less veterinary care and preventive treatments. Compared to a general average of more than two antihelmintic treatments per year for other dogs, the shepherd dogs in this study received only 0.5 treatments per year. Although the prevalence of intestinal parasites in dogs with owners was lower than in shelter dogs, the percentage of infested animals was significant (23.8%). Dogs, even if they are raised inside the house, are regularly walked by owners in public places and are in close contact with other dogs (including free-ranging dogs) (11).

In a study conducted in Italy, *Riggio et al.* (2013) investigated 239 dog feces, and in 36 of them, there was a parasitism with various gastrointestinal nematodes, with a general prevalence of 31%. The examined dogs were infested with *Toxocara canis* (13.0%), *Toxascaris leonina* (1.7%), *Trichocephalus vulpiscus* (3.3%), *Ancylostoma caninum* (2.0%), *Uncinaria stenocephala* (1.25%), *Strongyloides stercoralis* (0.8%), *Angiostrongylus vasorum* (0.4%), *Dipylidium caninum* (1.25%), *Taeniidae* (0.4%), *Giardia duodenalis* (3.8%) and *Cystoisospora spp.* (7.5%) (23).

In Tirana, Albania, *Shukullari et al.* (2015) analyzed 602 samples from dogs with owners, who presented themselves for proper medical advice in veterinary practices. Out of the 602 pets, 245 dogs (40.7%) were positive for at least one type of endoparasite, of which 180 (29.9%) were positive for protozoa and 129 (21.9%) for nematodes. At least 14 endoparasites have been identified. The most common were *Giardia* (26.4%), *Trichocephalus* (9.5%), *Toxocara* (8.0%), *Ancylostomatidae* (7.1%), *Cystoisospora ohioensis* (4.3%) and *Cystoisospora canis* (3%) (24).

In other European cities, epidemiological studies reported prevalence from 3.6%, in Ireland (17), 11.1%, in Italy (13), 24.3%, in Hungary (6, 7) to 31.5%, in Poland (9).

In this study, there was a higher prevalence of parasitism in young dogs with the age between 0 and 6 months (63%) compared to other age groups. Other studies also highlight a higher prevalence of parasitism in young animals; such a study, conducted by *Dubna et al.* (2007), in which the prevalence of *Toxocara canis* parasitism in dogs under 6 months, from two public shelters was 22.6% and 45.2%, respectively (5). These results are similar with those obtained in other studies, in Netherlands (3, 19).

Global research has now established that animals from public shelters are more positive than serving, guard or hunting animals, with owners. Among all the investigated parasites in other studies, with flotation methods, *Ancylostoma*

caninum has been identified as the main parasite in community dogs in the US public shelters (1, 10, 14).

Some authors (2) consider that an improvement of shelter conditions influences the number of infected animals. It was observed that, in shelters with better conditions in maintenance, the prevalence of parasitism decreased. Thus, cleaning the cages with pressurized water, maintaining a lower concentration of animals and a periodic veterinary control followed by animal deworming are measures that lead to a decrease in parasitic population. Also, cemented flooring and periodic cleaning are preventive measures that provide a lower prevalence of parasitism.

The nematode eggs are very resistant to extreme environmental conditions and remain with the capacity to infest for many years. Because there are no practical ways to reduce the number of eggs in the environment, preventing initial contamination is the most important aspect in preventing parasitism. This can be done by eliminating patented infestations in dogs and cats, preventing pets from spreading the eggs in public areas, but also through hygiene and public education (20).

Conclusions

The prevalence of nematodes in fecal samples from dogs with owners, in descending order, was: *Trichocephalus vulpis* (42.85%), *Toxocara canis* (36.73%), *Ancylostoma caninum* (14.28%), *Toxascaris leonina* (4.08%) and *Strongyloides stercoralis* (2.04%).

In shelter dogs, the following prevalence was recorded: *Ancylostoma caninum* (97%), *Toxascaris leonina* (32%) and *Toxocara canis* (19%).

The occurrence of parasitism was found to be high among the age group of 0-6 months and in large breeds and mixed breeds.

The most common monoparasitism was the one with *Ancylostoma caninum* (25%), found in the public shelter.

In the case of multiparasitism, the most frequent combination was *Ancylostoma caninum* and *Toxascaris leonina* (11.5%).

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STUDY ON THE VARIATION OF WHITE BLOOD CELLS COUNT IN DIGESTIVE DISORDERS IN DOGS

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Summary

The aim of this study was to evaluate the association between the magnitude of the leukocyte quantitative changes and the primary disorders of the digestive system in which they occurred. The study was performed by analyzing the complete blood count (CBC) from 45 dogs diagnosed with digestive disorders. The disorders of the digestive system in which the presence of leukocytosis was found more frequently were represented by: non-specific gastroenteritis (32%), acute pancreatitis (29%), inflammatory bowel disease (24%), chronic hepatitis (9) and other digestive disorders in proportion of 6%. The magnitude of leukocytosis was moderate in chronic hepatitis, inflammatory bowel disease and acute pancreatitis, while in non-specific gastroenteritis the increase in leukocyte count was of small magnitude. In this study, the digestive diseases that developed leukopenia in dogs, with the highest prevalence was parvoviral enteritis (72%), followed by non-specific gastroenteritis (14%) and other digestive disorders(14%).

In conclusion, non-specific infections and inflammations of the digestive tract are more frequently accompanied by moderate leukocytosis, while specific enteritis lead at least in the onset to leukopenia.

Keywords: white blood cell, leukocytosis, leukopenia, digestive disorders, dog

Haematological analysis of peripheral blood, assesses the production of a sufficient number of mature cells on the three major haematopoietic lines (myelocytic series, erythrocyte, megakaryocyte series) and if the exponents of the three series (erythrocytes, leucocytes, platelets) have normal morphology. Leucocyte hematological changes through multifactorial pathophysiological mechanisms may put the clinician in difficulty in establishing the etiologic diagnosis, requiring complementary methods of investigation (2, 4, 6).

Hemoleucogram is a basic screening test, being one of the most frequently required laboratory tests, and is often the first step in establishing the hematological status and diagnosis of various haematological and non-haematological conditions (4, 5, 6).

Leukocytes are inflammatory cells, and changes in the leukogram are mainly used to identify the presence of inflammatory disease and characterize inflammation as to severity and type. The leukogram is not highly sensitive in detecting mild, focal, or chronic inflammation; therefore, a normal WBC count and Diff does not exclude inflammatory disease from the diagnosis (4, 6).

An abnormal leukogram usually leads to identification of a pathologic process (e.g., inflammation), but not to establishment of a specific diagnosis. Interpretation of leukocyte abnormalities into a process coupled with clinical findings, however, may lead to a diagnosis (4).

The aim of this study was to evaluate the magnitude of leukocyte changes and the association between leukocyte changes and primary disorders of the digestive tract in which they occurred, in order to facilitate a more rapid diagnosis of digestive disorders.

Materials and methods

In this paper were analyzed the haemograms from 45 dogs, diagnosed with digestive disorders, aged between 5 months and 14 years old. All dogs included in this study were brought by the owners for clinical examination at the University Veterinary Clinic from Timișoara.

The inclusion criteria in this study were: haemograms performed at the time of diagnosis in dogs with digestive diseases; the number of leukocytes greater than 17,000 / μl or less than 6,000 / μl . The exclusion criteria were represented by: haemograms from dogs that presented other pathologic conditions associated; haemograms from dogs that have been treated with drugs until the etiological diagnosis has been established.

Blood samples were collected from the antebrahial cephalic vein or safen in EDTAK₃ tubes. The haemoleucogram was performed within two-four hours after blood sampling, and the blood smears were stained immediately after blood samples were taken, by Diff Quick method.

Haemograms were performed using two automatic haematology devices (ProCyte DX and Advia 2120i) by flow cytometry, cytochemistry and spectrophotometry.

Individual leukocyte counts were processed in mean values depending on the primary disease that generated the leukocyte change, using the SPSS 20 statistical processing program.

Results and discussions

Of the total of 45 patients with digestive diseases and leukocyte changes, 77.7% (n=35) had leukocytosis and only 22.3% (n=10) had leukopenia. Disorders of the digestive system that have associated more frequently with leukocytosis were: non-specific gastroenteritis (32%), acute pancreatitis (29%), inflammatory bowel disease (24 %), and chronic hepatitis (9 %) (Fig. 1).

In terms of the absolute number of white blood cells, moderate leukocytosis was found in chronic hepatitis, intestinal inflammatory disease and acute pancreatitis, whereas in non-specific gastroenteritis, the increase in leukocytes count was of small magnitude (Fig. 2).

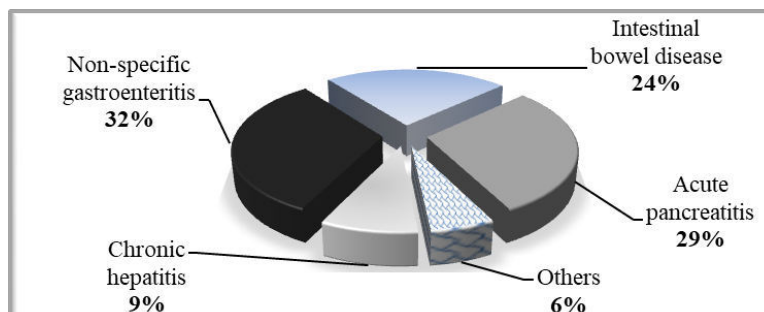


Fig. 1. Prevalence of leukocytosis depending on the digestive system disorders

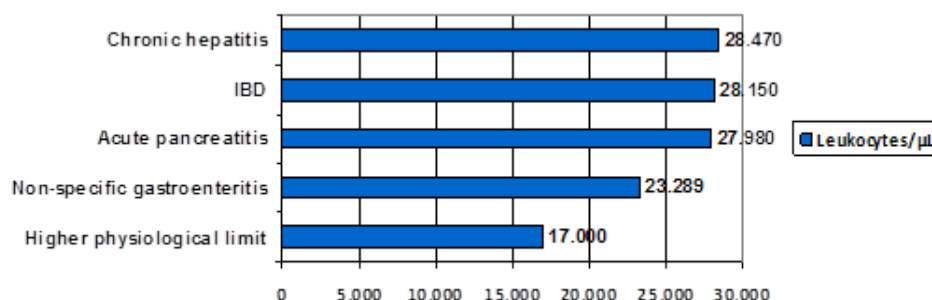


Fig. 2. Average leukocyte counts in digestive disorders that have evolved with leukocytosis

In general, the increase in the number of leukocytes in the main digestive diseases that evolved with leukocytosis was due to the increase in the absolute number and the percentage of neutrophils (Fig. 3). It can be seen that the magnitude of neutrophilia is directly related to the absolute number of leukocytes, while the percentage of lymphocytes is inverse proportional to the magnitude of leukocytosis. For the other white blood cells counts there were no changes of clinical significance over the physiological values.

The increase of leukocytes count that were found at dogs in this study could be explained by an increase in production and release of bone marrow neutrophils that exceeds consumption from the inflammatory lesion (3, 4). The changes in leukocytes count that were found in this study are consistent with other clinical trials. Thus, data from the literature reveal that acute pancreatitis causes severe neutrophilic leukocytosis, being the consequence of inflammation and necrosis of peripancreatic tissues resulting from the activation and release into the interstitial space of pancreatic enzymes (3, 5). With regard to chronic liver disease,

in a study by Elhiblu et al. (1) on 140 dogs, of which six were diagnosed with cirrhosis, leukocytosis with left deviation of Arneth's index associated with decreased lymphocyte and platelets, was observed.

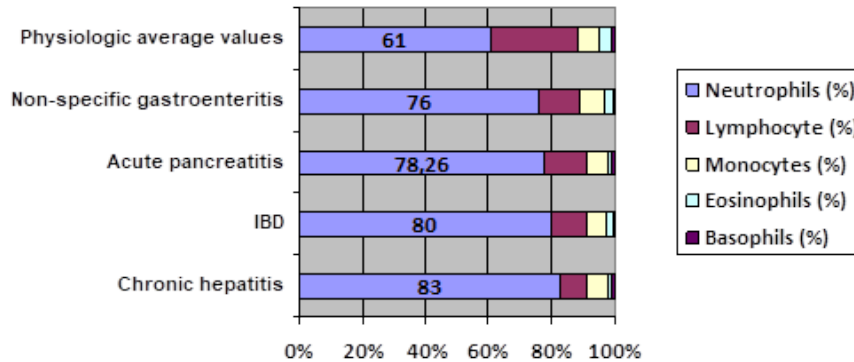


Fig. 3. The mean values of the leukocyte formula in digestive disorders that have evolved with leukocytosis

The leucogram changes obtained in this study are consistent with the results obtained by Mercedes et al. (2) who observed a significant difference in the number of monocytes in dogs with intestinal inflammatory disease compared to a normal group. Also, in dogs with inflammatory bowel disease, non-specific and specific haematological changes have been frequently described, such as neutrophilia and monocytosis, reflecting chronic, active inflammation (2).

Of the digestive system diseases that evolved with leukopenia in the patients in this study, the highest prevalence was parvoviral enteritis (72%), followed by acute pancreatitis and non-specific gastroenteritis, both with 14% (Fig. 4).

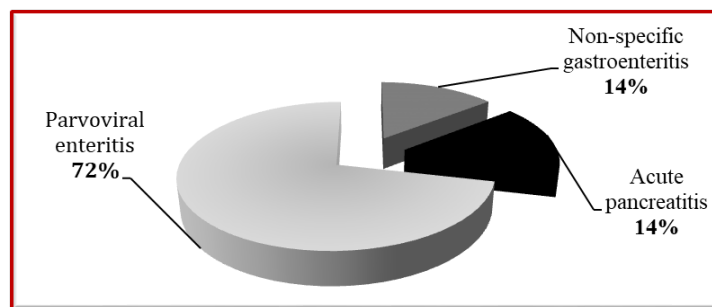


Fig. 4. Prevalence of leukopenia in digestive disorders

In absolute value the magnitude of leucopenia was small in the case of non-specific gastroenteritis, moderate in parvoviral enteritis and severe in the evolution of one case of acute pancreatitis in which the leukocyte count dropped below 1.000 / μl of blood (Fig. 5). In general, the reduction in white blood cells count was mainly due to the decrease in absolute neutrophil count and, to a lesser extent, due to the reduction in the number of other white blood cell counts.

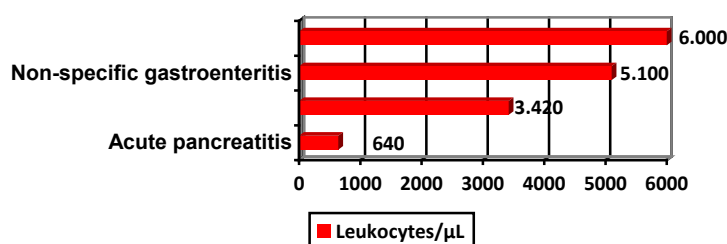


Fig. 5. Average leukocyte values in digestive disorders that have evolved with leukopenia

In parvoviral enteritis, there are several mechanisms through which leukopenia can occur with neutropenia, and the first is that the virus is cytotoxic to haematopoietic stem cells. Another mechanism would be a depletion of leukocyte precursors in the bone marrow maturation and storage compartment, due to endotoxemia as a result of gastrointestinal necrosis (5). In the case of nonspecific gastroenteritis and pancreatitis, leukocyte count reduction can be attributed to extra-vascular leukocyte consumption, which exceeds production, maturation and leukocyte release from haematopoietic organs (3, 4).

Conclusions

Non-specific infections or inflammations of the digestive system are more frequently accompanied by moderate leukocytosis, while specific enteritis lead, at least in the onset of leukopenia.

The onset of severe infections and inflammations is more commonly associated with neutrophilic leukopenia.

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RESEARCH ON FELINE HERPESVIRUS INFECTION

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Summary

The presence of feline herpesvirus infection in a cat population was demonstrate using a commercial kit – Fluo FELINE HERPES VIRUS Agrolab. From 20 serum samples which are tested, 8 samples were positive. It is well known that subclinical cats, which are serologically positive, are carriers and spread the virus, contributing to the persistence of virus in animal population.

Keywords: cat, herpes virus, serum

Feline herpesvirus type-1 is an infectious disease that causes feline 7 viral rhinotracheitis (FVR). As with other herpes viruses, the virus is very species specific, and is only known to cause infections in domestic and wild cats (1, 4, 5, 11, 17, 18, 20). The virus can infect cats of all ages. Feline Viral Rhinotracheitis is a major cause of upper respiratory disease in cats, and is the most common cause of conjunctivitis (inflammation of the tissues surrounding the eye, especially the lining of the lids and the third eyelid). A cat becomes infected with this virus by direct contact with virus particles (8, 9). The virus is excreted in saliva and in discharges from the eyes and nose of an infected cat (4, 5, 10, 12, 20). Therefore, an infection occurs when a susceptible cat comes into direct contact with an infected cat, or comes into contact with inanimate objects (called '*fomites*') that have been contaminated with viral particles (13, 15, 16, 19). The typical symptoms of FVR involve the nose, throat and eyes, and include sneezing, nasal congestion, conjunctivitis (inflammation of the tissues that line the eyelids and surround the eyes), excessive blinking, and discharges from the eyes and nose that range from clear and watery to thick and purulent -containing pus (2, 19). The virus may also cause *keratitis*, or inflammation and infection of the cornea, leading to corneal ulcers (3, 6, 7, 16). In chronic or severe infections, the keratitis can lead to corneal scarring or chronic ``dry eye`` - *keratoconjunctivitis sicca* or KCS (5, 8, 9).

Other non-specific symptoms may include fever, lethargy, anorexia or poor appetite, and enlarged lymphnodes (9, 20).

Materials and methods

The present study included 20 short and long-haired domestic cats belonging to six different breeds (Persian, British short hair, Birmingham, American short hair, Siamese and European) presented at the Veterinary Clinic of the Faculty of Veterinary Medicine Timisoara, respectively at different private veterinary clinics in Timisoara, between January 2018 and May 2018.

Cats lived indoors and outdoors, with ages between 3 months and 7 years, and of both genders.

The cats taken in this study showed clinical signs of conjunctivitis or other ocular diseases associated with / without clinical signs of respiratory infections, and fever. After a clinical exam, associated with a detailed eyes and conjunctiva examination, the fluorescein test was used to highlight corneal lesions, that are unseen at usual examination.

The clinical and ophthalmologic examination was followed by sampling conjunctival secretion from the 20 cats which presented clinical signs of conjunctivitis. The samples were transported in the same day or within 24 hours at the laboratory, where they were prepared for immunofluorescence assay.

Fluo Feline Herpesvirus – Agrolab kit used in this study is based on detection of anti-IgG antibodies in serum or plasma of cats (4, 9, 20).

The samples from the cat are diluted in buffered saline and incubated on individual blades to allow the reaction between the antibodies from the tested cats to the inactivated FHV-infected cells together with the uninfected cells attached to the blade. The lamellae are then washed to remove unreacted serum proteins, and then a conjugate containing fluorescein-labeled anti-cat IgG antibodies is added. The positive reaction is expressed with a strong green fluorescence, representing the binding of the superficial protein to each infected cell. In the negative reaction, the cells show no fluorescence and appear in gray-green color. Using a yellow light filter, they appear in red, in contrast to the positive control. Test filters should be either green or yellow. By using the green filter, positive serum will appear in cells with a fluorescent greenish reaction. Using the yellow filter in the case of negative serum cells will look red in addition to the blue Evans dye. Positive dilution test 1:40, IgG titers at dilutions of 1:40 and above are considered to reflect an infection at an undetermined time. Positive dilution should be re-examined to determine their final titre, comparing the original samples with those subsequently taken from the same animal (4, 13, 14, 17).

Results and discussions

The studied cats' population was represented by cats with different ages - 13 of them were between 3 and 12 months old and 7 were adult cats (between 2 and 7 years old), 12 were females and 8 males.

Some of these cats (15) were vaccinated against feline rhinotracheitis, calicivirus infection and panleukopenia (Felocell CVR®, Virbac) and five of them

were vaccinated against feline leukemia virus infection (Leukocell 2®, Pfizer). None of the studied cats were vaccinated against *Chlamydophila felis* infection.

In most cases (16/20), there were no clinical signs of systemic disease. Clinical signs of these cats included conjunctivitis (20/20), nasal discharge following a rhinitis (20/20), bronchopneumonia (4/20), anorexia (4/20) and fever (40°C) in 3 cats.

Conjunctivitis affected only one eye in 13 cases and in four cases was registered bilateral conjunctivitis. The prevalence of FHV-1 in cats throughout the world has been frequently reported and has different values between 5.9% and 12% (20).

If reference is made to owner-sick cats, FHV-1 was detected between 4.5% and 76.3% of the cats in Japan, the United States, Australia, and Italy (8, 20).

Based on these researches, the number of positive cats for FHV-1 in shelters was higher than the number of positive cats from cattery. The results are due to the fact that most of the cats in the shelters have been infected with FHV-1 and will remain subclinical carriers after recovery. At least 80% of them remain animals with latent infection and 29% of them eliminate the virus spontaneously.

Conclusions

This study has shown that many of the cats which living outside and inside remain latently infected with FHV-1.

These cats will be sources of infection for other cats, especially when they are in exhibitions or in veterinary hospitals.

A proper management, represent by cleaning and disinfection cats' space, a less stressful environment, avoid an overcrowding and designing appropriate cages is required in a cat shelter (exhibitions, hospitals).

Although vaccination against FHV-1 cannot prevent viral infection and the appearance of carriers, however, this may reduce the severity of the disease. Vaccination of the cats are necessary in shelters or in places with a large number of animals and to avoid the contact of healthy cats with cats with unknown vaccination history.

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MAGNETIC RESONANCE IMAGING FINDINGS IN TWELVE DOGS WITH HYDROCEPHALUS

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Summary

Hydrocephalus is defined as an excessive accumulation of cerebrospinal fluid (CSF) in the cranial cavity and dilation of the ventricular system. It is not a specific disease, but rather a multifactorial disorder with a variety of pathophysiological mechanisms. Is a common congenital or acquired neurological disorder in dogs, affecting both young and adults patients of any breed but frequent toy-breeds. Neurologic signs associated with hydrocephalus are variable and include a specific series of signs depending on the age of the patient. In young animals with congenital hydrocephalus we can usually see signs like restlessness, behavior problems and seizures, while older animals with acquired hydrocephalus can show signs such as blindness, circling and in severe cases patients often deteriorate to stupor or coma. The aim of this study was to evaluate the cerebral ventricular system morphology and to compare cases and make correlations between them using Low-field Magnetic Resonance Imaging (MRI). Therefore, medical records from the Imaging Center of the Faculty of Veterinary Medicine of Bucharest were reviewed in order to identify dogs diagnosed with hydrocephalus. Twelve dogs, 8 males (M) and 4 females (F) of different breeds, between five months and eight years old, with clinical, neurological signs were included in the study. The most common clinical sings were represented by seizures, ataxia and circling. Following the MRI examination, we were able to describe and classify the affection in order to make a differential diagnosis .Six cases were included in the category of congenital hydrocephalus; five cases showed acquired hydrocephalus and one idiopathic case. Due to the fact that clinical symptoms differ, using advanced imaging techniques is important in order to have a precise diagnosis and to classify the pathology. Therefore MRI is an essential tool for the detection of hydrocephalus because it has the best diagnostic volume and is a very sensitive method.

Keywords: magnetic resonance imaging, hydrocephalus, dogs

Hydrocephalus is a common congenital or acquired neurological condition present in dogs and cats, caused by an abnormal accumulation of cerebrospinal fluid (CSF), resulting in pressure on the brain. It is not a specific disease, but rather a disorder caused by several factors involving a variety of pathophysiological mechanisms (2, 7). Hydrocephalus is characterized by the anatomic relationship of the underlying disease process and abnormal pressure differences (3). Hydrocephalus can be categorised in two big different types; high pressure and normal pressure; CSF pressure measurements are rarely done in veterinary medicine, normotensive and hypertensive are descriptive terms used to further classify cases of hydrocephalus (4). High pressure hydrocephalus can be categorised in three different ways.

Depending on the location of the accumulated CSF can be internal or external hydrocephalus, which refer to increased fluid accumulation within the ventricular system and outside the ventricles, with an enlarged subarachnoid space respectively (1, 2). Referring to the flow of CSF can be communicating hydrocephalus which refers to an inability of the CSF to pass through the arachnoidal villi to get back into the blood stream; or non-communicating hydrocephalus which refers to obstruction of the CSF pathways within the interior of the brain or at the tentorial notch.

Aetiological, hydrocephalus can be divided into congenital or acquired forms (2, 7). In veterinary patients, congenital hydrocephalus is more common than acquired hydrocephalus. In a retrospective study of 564 cases of congenital hydrocephalus (5), 11 breeds were determined; Maltese, Yorkshire terrier, English bulldog, Chihuahua, Lhasa apso, Pomeranian, toybreed, Cairn terrier, Boston terrier, Pug, and Pekingese. The causes are diverse and include genetic factors, developmental anomalies, intrauterine or prenatal infection. Congenital hydrocephalus occurs secondary to a wide range of nervous system anomalies, including meningomyelocele, Chiari malformation, Dandy-Walker syndrome and cerebral hypoplasia (7, 8).

Obstructions may become apparent secondary to congenital stenosis of mesencephalic aqueducts or lateral apertures (2). Obstructions also can be found due to a variety of causes such as tumours, cysts, thromboembolism and developmental abnormalities. Secondary to decreased brain parenchyma there is compensatory hydrocephalus (ex vacuo). A decrease in parenchyma can occur following trauma, infarction or necrosis. Normal pressure hydrocephalus (NPH) is the enlargement of the ventricles of the brain without increased CSF pressure, which in turn leads to compression of the brain tissue.

Depending on the time of onset, hydrocephalus is divided into acute and chronic (2, 7). Acute hydrocephalus, rather than chronic, relatively compensated normotensive hydrocephalus, is most frequently associated with inflammatory disease, periventricular oedema, subarachnoid haemorrhage and increased intraventricular pressure (6, 7, 9).

Neurologic signs associated with hydrocephalus are variable and include a specific series of signs depending on the age of the patient. Animals with congenital hydrocephalus are often smaller than their littermates and morphological malformations include an enlarged head with persistent fontanelles and open cranial sutures are often evident. Hydrocephalic animals exhibit altered mental states ranging from depression to hyperexcitability, disturbed consciousness, visual and auditory impairment, incoordination, circling, seizures, as well as symptoms such as dilated and fixed pupils and blindness. While older animals with acquired hydrocephalus can show signs such as blindness, circling and in severe cases patients often deteriorate to stupor or coma. This study presents twelve cases of hydrocephalus in dogs, starting with clinical and neurological examination to imaging diagnosis using magnetic resonance imaging (MRI) investigation.

Material and methods

The study was conducted on twelve dogs, eight males (M) and four females (F) of different breeds (two French Bulldog, two Siberian Husky, one Pittbull Terrier, one Beagle, one Yorkshire Terrier, one Pekingese, one Shih-Tzu, one Pug and two half-breed dogs), between five months and eight years old. They were examined general and neurologically, and in four of them we performed X-ray examination. The dogs were evaluated at the Imaging Center of the Faculty of Veterinary Medicine of Bucharest. All patients underwent general anesthesia consisted in premedication with Butorphanol 0.3 mg/kg IV, induction with Propofol 4-5 mg/kg IV and maintenance with Isofluran and O₂. MRI studies of the head were performed with a Vet-MR Grande Esaote system. A standard spin echo pulse sequence protocol was used included: 3.5- 4 mm thick slice with a 0.5 mm interslice gap in the sagittal, transverse and dorsal plane for T1 and T2W images. Also fluid-attenuated inversion recovery (FLAIR) sequence in the transvers plane was performed. Also, in four cases Gadolinium was used as a contrast medium agent, with dose of 0.1 mmol/kg.

Results and discussions

MR images of the 12 dogs were analyzed and compared to establish a diagnosis. Normal CSF will appear T1 hypointense and T2 hyperintense to brain parenchyma and will have low signal on FLAIR. All cases had moderate to marked dilation of the lateral ventricles with variable degrees of dilation of the 3rd ventricle. In five dogs unilateral hydrocephalus was present by the accumulation of one of the lateral ventricles, three of them on the left ventricle and two on the right one. In eight dogs the 4th ventricle was considered within normal limits and in four cases dilated. Nine dogs were examined for presence of periventricular hyperintensity on the FLAIR sequences which was identified in four dogs.

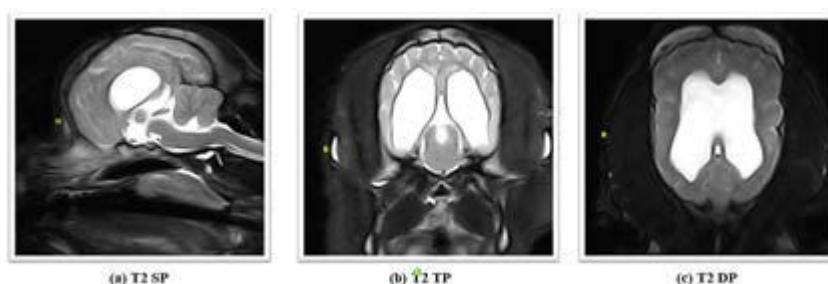


Fig. 1. French bulldog, M, seven months old, presented with ataxia and seizures, following the scanning, marked generalized hydrocephalus is seen on all image sequences. There is evidence of enlargement and accumulation of CSF in all four ventricles, but the lateral ventricles are the most prominent. Third ventricular dilation is also evident, which is best appreciated on the sagittal T2 image (a)

On reviewing T1-pre and postcontrast sequences, contrast enhancement was seen in three dogs with intracranial neoplastic processes. Six cases were included in the category of congenital hydrocephalus; five cases showed acquired hydrocephalus and one idiopathic case.

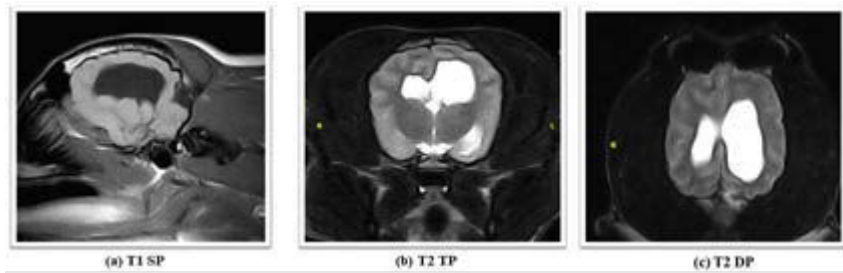


Fig. 2. Half breed, M, six years old presented with neurological deficit and seizures, following the scanning a ventricular asymmetry is observed, the left lateral ventricle is markedly distended (b,c)

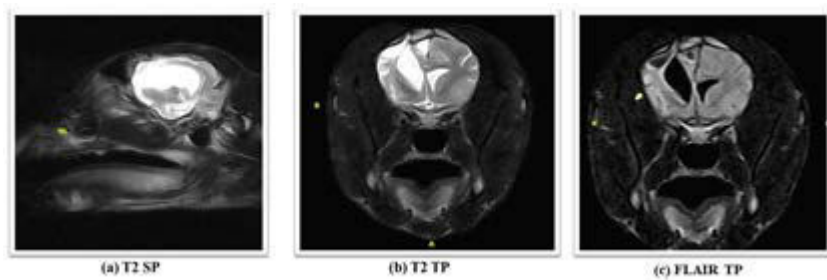


Fig. 3. Siberian Husky, F, one and half years old, presented with ataxia and circling on the right side, following the scanning an ventricular asymmetry is observed, the right lateral ventricle is moderate enlarged, also has evidence of cortical atrophy on the right cerebral hemisphere filled by CSF. At the right lateral ventricle margins there is a thin rim of hyperintensity best seen on the FLAIR sequence thought to represent interstitial edema (c)

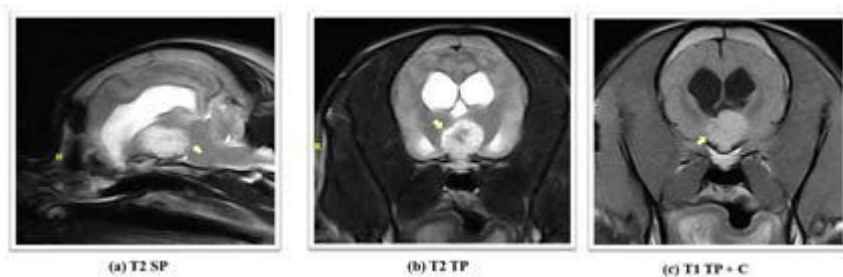


Fig. 4. French Bulldog, M, seven years old, presented with seizures and ataxia, there was discovered a large, well defined mass with contrast enhancement, that compressed the 3rd ventricle and causing partial obstruction and dilatation of lateral ventricles (c)

Conclusions

Following the MRI examination, we were able to describe and classify the affection in order to make a differential diagnosis. Six cases were included in the category of congenital hydrocephalus; five cases showed acquired hydrocephalus and one idiopathic case. Due to the fact that clinical symptoms are varied, using advanced imaging techniques is important in order to have a precise diagnosis and to classify the pathology. Therefore MRI is an essential tool for the detection of hydrocephalus because it has the best diagnostic volume and is a very sensitive method.

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PRELIMINARY STUDY ON THE PREVALENCE OF *DIROFILARIA IMMITIS* AND TREATMENT EFFICACY IN SHELTER DOGS

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Summary

Due to an increased number of dogs diagnosed with dirofilariosis in a dog shelter from Timisoara, a protocol was introduced in order to detect and treat dogs with *Dirofilaria immitis*. A number of 257 samples were taken in EDTA vacutainers and consecutively examined by drop test and Knott modified test. Furthermore, certain characteristics, such as sex, age, length of fur and body weight were evaluated. Blood analysis determined that 23.3% of dogs were positive. They were subsequently treated with doxycycline, followed by topical imidacloprid / moxidectin solution. So far 30 dogs had a 6 month waiting period after having been treated; those were tested again with an Idexx SNAP Heartworm RT Test. The post-treatment test revealed that a number of 22 dogs were negative and 8 dogs tested positive again. The treatment efficacy rate was 73.3 % which, taking the alternative treatment schemes and their benefits and risks into consideration, can be stated as satisfying. The risk group is represented by older, high-weight, male dogs with short fur. The evaluation of the characteristics allows a better understanding of risk groups in dogs.

Keywords: *Dirofilaria immitis*, dirofilariosis, shelter dogs

Cardiovascular dirofilariosis is caused by a nematode of the family Onchocercidae, called *Dirofilaria immitis*. A variety of different mammals and even reptiles can be infected. The disease is transmitted by mosquitoes during the feeding on the host animal and is endemic in several European countries including Romania, with the number of cases gradually increasing (4, 5, 6).

When the mosquito penetrates the host's skin, it releases larvae of the third stage into the body where they migrate through tissue into the blood stream and evolve into fourth stage larvae. With the blood stream they migrate towards the heart where they will have their final transformation into adult heartworms.

On average, one mosquito bite will release an approximate number of 50 larvae into the body. Around 15-35 will reach the final transformation and become adult heartworms (7). Although dirofilariosis often has an asymptomatic progress, it may affect the cardiopulmonary system, lead to irritation in the vessels or even cause obstruction. It is therefore of importance to treat the animal as soon as possible (9).

There are different approaches to treat this disease, the most commonly used one is the slow-kill method using imidacloprid/moxidectin or ivermectin and doxycycline which targets the bacterial endosymbiont *Wolbachia*, or a method using Melarsomine dihydrochloride (Immiticide®, Merial). The latter one is the only

approved and recommended treatment plan by the American Heartworm Society, however using this method movement-restriction is vital and costs are significantly higher compared to the slow-kill method. Melarsomine injections have to be performed with great caution since a multitude of side effects might occur including severe pulmonary thrombosis (1).

McCall et al. (2014) conducted a study on dogs with experimentally-acquired infection and treated with doxycycline for 1 month at 20 mg/kg/day. Worm survival was evaluated at 12 and 13 months post-infection (p.i.) and results suggested that treatment had a slow-kill, adulticidal effect. Doxycycline was administered in experimentally infected dogs during the first month postinfection, and no live worms were observed at necropsy, indicating 100% efficacy of treatment against infective larvae. Antibiotic treatment administered starting at 40 days p.i. revealed 98.4% efficacy. When treatment was initiated 65 days p.i., efficacy against juvenile worms was 69.6% (9, 10, 11).

Another effective method is the use of ivermectin/doxycycline combination protocols with adulticide effect on naturally infected dogs (2, 8, 13).

Microfilaricidal effect of imidacloprid 10%/moxidectin 2.5% (Advocate®, Bayer Animal Health) was evaluated in a study on sixteen animals, eight infected with *D. immitis* and eight with *D. repens*. Results showed high microfilaricidal efficacy (99.97% and 100% for *D. immitis* and *D. repens*, respectively) of a single dose of Advocate® in naturally infected dogs (3).

The aim of the study was to evaluate the prevalence of *D. immitis* and to assess the efficacy of a slow-kill method with doxycycline and imidacloprid /moxidectin treatment.

Materials and methods

A number of 257 of blood samples were collected from dogs in a private shelter in order to determine the presence of circulating microfilariae. The dogs were kept in outdoor kennels and were therefore constantly exposed to mosquitoes.

The blood samples were taken in EDTA vacutainers and the amount of blood drawn was between 0.3 ml and 1 ml. Furthermore, certain characteristics, such as sex, age, length of fur and body weight were evaluated. Samples were examined in the Parasitology Laboratory of the Faculty of Veterinary Medicine Timisoara. Following up this sampling day, every dog older than 2 years of age that has entered the shelter was sampled and the blood was examined by the veterinarian associated with the shelter.

Samples were examined by drop test and Knott modified test. All positive dogs received treatment and 6 months later were tested again.

Post-treatment blood examination was done using the SNAP Heartworm RT Test (SNAP(®) Heartworm RT Test; IDEXX Laboratories), which reacts in the presence of antigens released by the adult heartworm (Fig. 1). This method was chosen as it is more reliable and can test the adulticide effect of the treatment.

The treatment was effectuated with doxycycline and imidacloprid/moxidectin topical solution Advocate® (Bayer Animal Health). For 21 days doxycycline was administered in the dose of 10 mg/kg bodyweight q.d. On the last day of doxycycline administration imidacloprid and moxidectin were applied as spot-on formula. The application was repeated after one month and final imidacloprid and moxidectin administration was done after yet another month.

Dogs which test positive again after the 6 month waiting period will be treated again with the same treatment scheme.

Results and discussions

There were tested 257 blood samples, of which 60 dogs were found positive, which corresponds to a prevalence of 23.3 %. Post-treatment blood tests have been done on 30 dogs and it was found that 8 (26.6 %) were still positive and 22 were negative(73.3 %). The 22 negative dogs were considered successfully treated.



Fig. 1: Idexx Antigen Test

The gender distribution of the 60 positive dogs lays by exactly 40 % female and 60 % male while the gender distribution of the total number of dogs is 54.5 % female and 45.5 % male. The lower number of male dogs in the population, yet their higher prevalence in the distribution of positive dogs indicates a predisposition for male dogs. Other studies from Romania presented that males and dogs older than 2 years have a predisposition for infection with *D. immitis* (12).

Since the ages of the dogs can only be estimated due to their backgrounds, most of them coming from the streets or having been abandoned, a system of 3 age ranges has been introduced (Table 1).

Table 1

Distribution according to age

Group	Age	Distribution among positive dogs	Distribution among the whole population
A	2 - 5 years	38.3%	43.2%
B	6 - 8 years	41.7%	45.5%
C	> 9 years	20%	11.3%

Most dogs tested positive are in age group B, followed by age group A with 25 respectively 23 dogs. In the age group C only 12 dogs were positive. The

percentage distribution of positive dogs is 38.3 % in group A, 41.7 % in group B and 20 % in the group C. The general age distribution among the whole population was 43.2 % in group A, 45.5 % in group B and 11.3 % in group C.

A shift towards age group C can be noted, however considering that the dogs in this group have had been exposed to the risk of infection for more years than the other two age groups, the higher incidence rate is no surprise.

Hair length was classified into 3 groups, presented in Table 2 and Fig. 2, 3, and 4. According to the length of fur, the general repartition of dogs lies by 24.9 % - short hair, 53.3 % - medium long hair and 21.8 % - long hair.

Table 2

Distribution according to length of fur

Group	Length of fur	Distribution among positive dogs	Distribution among the whole population
A	short fur	35%	24.9%
B	medium long fur	55%	53.3%
C	long fur	10%	21.8%



Fig. 2. Representation of a short haired dog, mixbreed



Fig. 3. Representation of medium long haired dog, mixbreed



Fig. 4. Representation of a long haired dog, mixbreed

Taking into account the length of hair classification, the following prevalence of positive dogs was highlighted: 21 dogs with short hair (35 %), 33 dogs with medium long hair (55 %) and only 6 dogs with long fur (10 %).

The lower prevalence of dirofilariosis for long haired dogs and the higher prevalence for short haired ones support the logical assumption that long hair is hindering for mosquitoes and therefore slightly decreases the risk of infestation.

In order to determine whether bodyweight influences the likeability of an infection, 3 bodyweight groups were considered (Table 3).

Table 3

Distribution according to bodyweight

Group	Bodyweight	Distribution among positive dogs	Distribution among the whole population
A	< 10 kg	8%	18.3%
B	11 - 25 kg	55%	54.5%
C	> 26 kg	37%	27.2%

According to the bodyweight, the positive dogs were distributed as follows: group A - 5 dogs, group B - 33 dogs and group C - 22 dogs which represent 8 %, 55 % and 37 % respectively. The distribution of the dogs, following bodyweight classification, was 18.3 % in group A, 54.5 % in group B and 27.2 % in group C.

The comparison shows a low number of light-weight dogs being positive, while the number of heavy dogs was higher, suggesting an increased infectivity for heavy dogs. However further investigation could be made on whether the higher infectivity could be explained by the dogs in group C possibly being overweight and therefore being less energetic rather than being of a big size. Less movement could lead to a higher attractiveness for mosquitoes. Following this thesis, large breed dogs do not have a higher infectivity rate, only heavy weight dogs do.

However, the housing system has to be taken into consideration. Other studies (12) showed that stray dogs and dogs kept outside have a predisposition to develop dirofilariosis due to frequent exposure to mosquito bites.

Conclusion

Evaluation of all collected data revealed a prevalence rate of 23.3 %.

The treatment efficacy rate was 73.3 % which, taking the alternative treatment schemes and their benefits and risks into consideration, can be stated as satisfying.

The risk group is represented by older, high-weight, male dogs with short fur.

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PCR DIAGNOSIS OF FASCIOLA HEPATICA IN INTERMEDIARY HOSTS-SNAILS COLLECTED FROM THE ENVIRONMENT

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Summary

Fasciolosis is a zoonotic trematodosis of great importance, transmitted to definitive hosts, such as herbivorous mammals and even humans through contaminated water or green grass. The causative agents of this disease are *Fasciola hepatica* or *Fasciola gigantica*.

The aim of this study was to identify the juvenile life cycle stages of this trematode in the *Lymneidae* snails which act as intermediate hosts. This was done in order to establish the *Fasciola*-contaminated areas in the context of animal movement as help in the parasitic control of this disease. 123 (51.3%) of the total number of collected snails were positive for the presence of *F.hepatica* cercaria, confirmed by PCR. The prevalence of the disease, respectively of the juvenile forms ranged according to the collection sites of the snails from 40 to 70.6. Two sample sites were negative. The current study brings back aspects of a very important disease, of great economical interest in the pathology of farm animals and it is the first that we know of, conducted in Romania.

Keywords: *Fasciola hepatica*, intermediary host, *Galba truncatulla*, PCR

Fasciolosis is a trematodosis transmitted to herbivores through contaminated water or green vegetables (9, 10). The aetiological agents of this disease are *Fasciola hepatica* and *Fasciola gigantica*.

Fasciolosis is a severe disease of animals from both rural and urban environments, worldwide, leading to great economic losses due to low productivity and confiscation of animal organs upon slaughtering (14, 15). Hepatic trematodes cause severe pathological signs in sheep and cattle. Their life cycle is complex and it implicates the snail as intermediary host. Snails are mainly found around water sources and through their role as intermediary host they contribute to the infection of the water plants and water-surrounding vegetation with cercaria, which later encyst and become metacercaria (11, 16).

Two thousand million dollars are lost annually due to low productivity caused by helminth infestations. In addition, fasciolosis is also seen as an emerging disease in humans as well. WHO reports have anticipated a number of 180 million people exposed to the risk of infestation and 2.4 million people that are already infested with *Fasciola* (11, 13). Presently, there are no commercially available, efficient vaccines (2, 4, 5, 6, 7, 12, 17).

The aim of this study was to identify juvenile forms from *Fasciola hepatica*

life cycle found in the intermediary hosts-the *Lymneidae* snails, in order to establish the possible areas, infested with the parasite, in the context of animal movement. This report is meant to act as support in the parasitic control of this diseases.

Materials and methods

Biological material sampling

The study was performed between the months April-June 2018, in eight different locations from the territories of Timiș and Arad counties (Table 1, Fig. 1).

Table 1

Areas of snail-collection

Sampling location	No. of collected snails
Location 1	32
Location 2	50
Location 3	47
Location 4	54
Location 5	40
Location 6	17
Location 7	-
Location 8	-
Total	240

Key: locations 1-8 = Bodo, Cladova, Jupani, Mănăștiur, Sudriaș, Surducu Mic, Săceni – Timiș, Vinga – Arad.

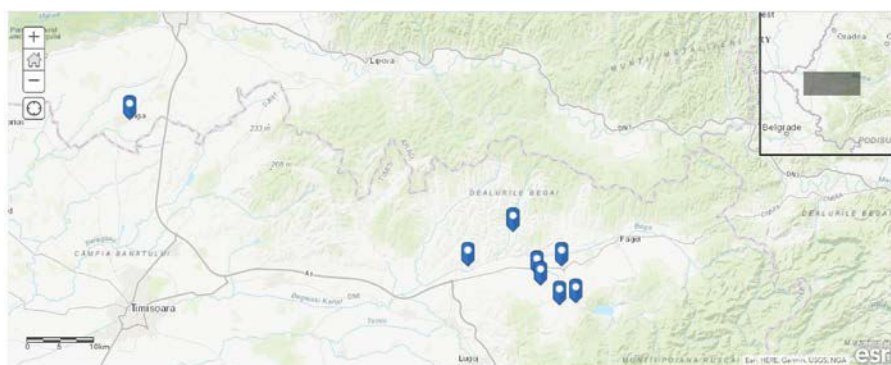


Fig. 1. Areas of snail-collection

The snails were collected in plastic containers, along with water from the collection spot and they were transported to the department of Parasitology and Parasitic Diseases of the FVMT, where they were relocated and separated individually in transparent plastic containers. The snails were kept at room-temperature for 7 days, in order for the possible cercaria to be released into the water from the glass. The presence of cercaria was observed by examining the water from the container with the magnifying glass.

Molecular Protocol

a. Parasitic DNA extraction

The first stage of molecular analysis was isolation of the parasitic genomic DNA, performed with the help of the PureLink® Genomic DNA Mini Kit (INVITROGEN®) kit. The kit contains K proteinase, A ribonuclease, lysis buffer, "PureLink® spin columns" with collector tube, buffer wash solution 1 and 2, "PureLink® Genomic Elution Buffer" and 2 ml collector tubes.

b. Polymerase chain reaction (PCR)

The PCR reaction was performed according to the technique described by Cucher et. al. (3).

Two primers have been used, as follows:

- forward primer - FhCO1F (50-TATGTTTTGATTTTACCCGGG-30),
- reverse primer - FhCO1R (50-ATGAGCAACCACAAACCATGT-30)

c. Amplicon analysis

The control of the amplicons was done through horizontal electrophoresis in submerged system of 1.5% agarose gel electrophoresis at 120 V and 90 mA, for 60 minutes.

Results and discussions

Following morphologic investigations of the snails collected from the environment, it was established that they belonged to the *Lymnaeidae* family, *Galba (Lymnaea)* genus, *G. truncatula* species based on the visible morphological characteristics of the shell, presented by Hurtrez-Boussès (8) (Fig. 2 and 3). The measurements were performed under a stereomicroscope.

The height of the shell was 5-10 mm and the width 2.5-6 mm. The maximum length of the shell is approx. 12 mm. The columella is folded, the tentacles are wide and have a wide base and the eyes are small. The coating of the mantle is covered in less pigmented, whitish spots, giving a pale, transparency to the shell of the living specimens.



Fig. 2. Morphological characteristics of the *G. truncatula* snail shells, collected for the study

The collected and identified snails were introduced in transparent containers with water and left to rest in light and at room temperature. The water in the container was checked every 24 hours in order to identify the cercaria that might have been released from the intermediary host.

Following DNA identification, extracted from the cercaria collected from the water and subjected to the polymerase chain reaction as described by Cucher et. al (7), it was noticed that not all snails were positive for parasitism with juvenile forms of *Fasciola hepatica* (Table 2).

Table 2.
Results of PCR performed on the cercaria released from the snails subjected to study

Collection spots	No. of collected snails	No. of snails with cercaria	No. of samples positive after PCR	Snails positive on PCR (%)
Location 1	32	22	15	46.9
Location 2	50	31	24	48
Location 3	47	29	25	53.2
Location 4	54	35	31	57.4
Location 5	40	24	16	40
Location 6	17	17	12	70.6
Location 7	-	-	-	-
Location 8	-	-	-	-
Total	240	158	123	51.3

Key: locations 1-8 = Bodo, Cladova, Jupani, Mănăștiur, Sudriaș, Surducu Mic, Săceni – Timiș, Vinga – Arad

Out of 240 total snails, 158 were positive for cercaria while the others were negative following PCR. However, only 123 out of 158 (51.3%) had *F. hepatica* cercaria.

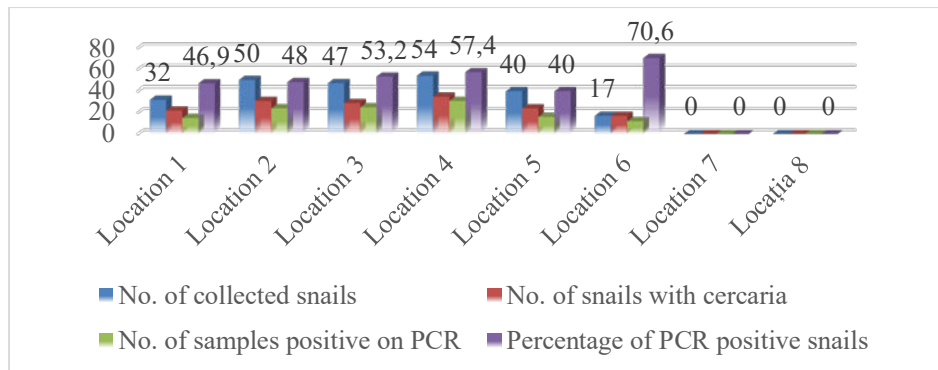


Fig 2. Graphic representation of PCR results from the cercaria released from the snails subjected to study

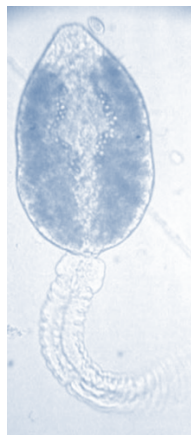


Fig. 3. *F. hepatica* cercaria that had DNA extracted for PCR



Fig. 4. 1,5% agarose gel electrophoresis image of the amplicons resulted from amplification of cercaria DNA with specific FhCO1F/FhCO1R primers

The interpretation of the results is based on the fact that in well no. 1 there is the molecular marker 100 pair-bases and in well no. 2- the control-DNA sample, positively amplified from *Fasciola hepatica* adults. In well no. 3 there is liquid from the collection container and in well no. 4 the DNA of cercaria collected from the

liquid in which the snails were immersed, amplified with specific primers for trematodes FhCO1F/FhCO1R described by Cucher et.al, at approx. 405 bp.

Snails were identified in only 6 out of 8 locations under study. The identified snails belonged to the species *Galba truncatula*, *Limnaea stagnalis* and *Stagnicola palustris* but only *G. truncatula* snails were examined. The results obtained from every location are presented in Fig. 5-10.

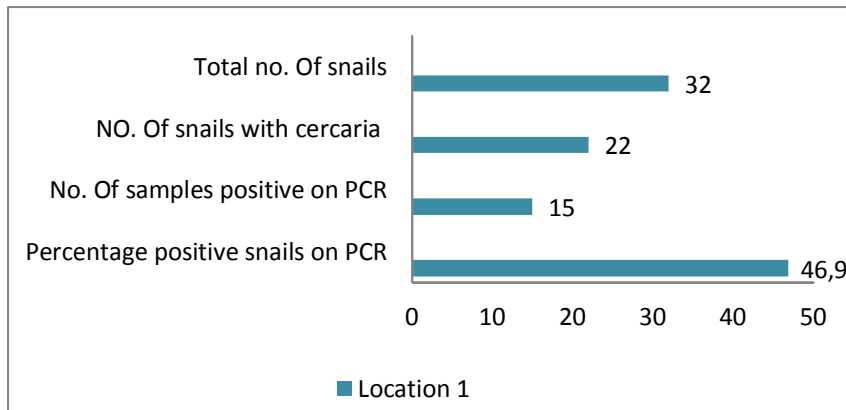


Fig. 5. Graphic representation of PCR results obtained from cercaria released from snails collected from Bodo

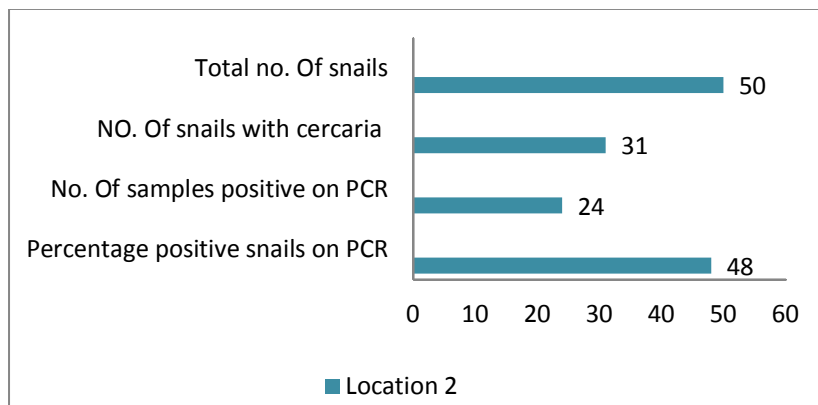


Fig. 6. Graphic representation of PCR results obtained from cercaria released from snails collected from Cladova

Thirty two snails were collected from location 1, Bodo, out which 22 were infested with cercari, but only 15 were *F. hepatica*, showing a prevalence of 46,9%.

Fifty snails were collected from location 2, Cladova, out of which 31 were infested with cercaria, but only 24 were *F. hepatica*, showing a prevalence of 48%.

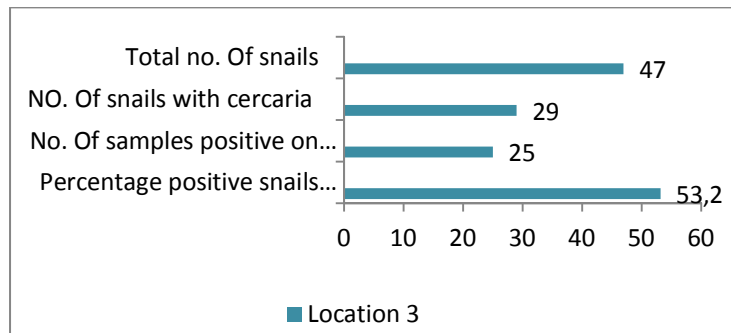


Fig. 7. Graphic representation of PCR results obtained from cercaria released from snails collected from Jupani

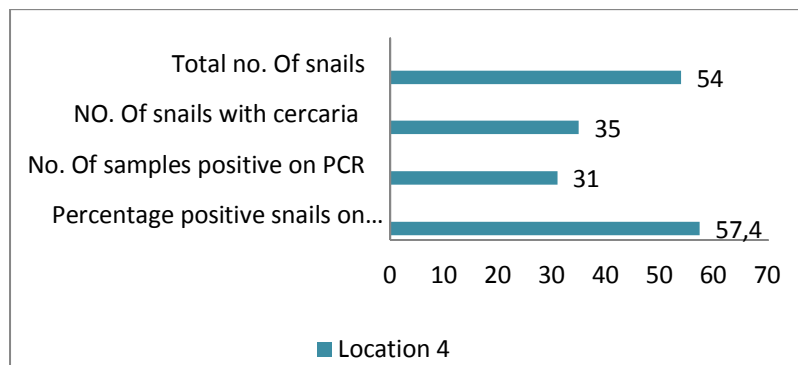


Fig. 8. Graphic representation of PCR results obtained from cercaria released from snails collected from Mănăștiur

Forty seven snails were collected from location 3, Jupani. Only 29 were positive for cercaria, but only 25 were *F. hepatica*, representing a prevalence of 53,2%.

Fifty four snails were collected from location 4, Mănăștiur Only 35 were positive for cercaria and 31 were *F. hepatica*, representing a prevalence of 57,4%.

Forty snails were collected from location 5, Sudriaș. Twenty four were positive for cercaria but only 16 were *F. hepatica*, representing a prevalence of 40%.

Seventeen snails were collected from location 6, Surducu Mic. All of them

were positive for cercaria, but only 12 were *F. hepatica*, representing a prevalence of 70,6%.

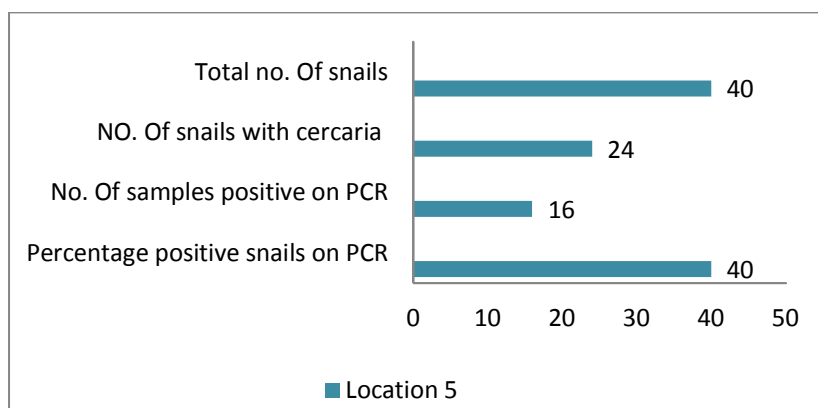


Fig. 9. Graphic representation of PCR results obtained from cercaria released from snails collected from Sudriaș

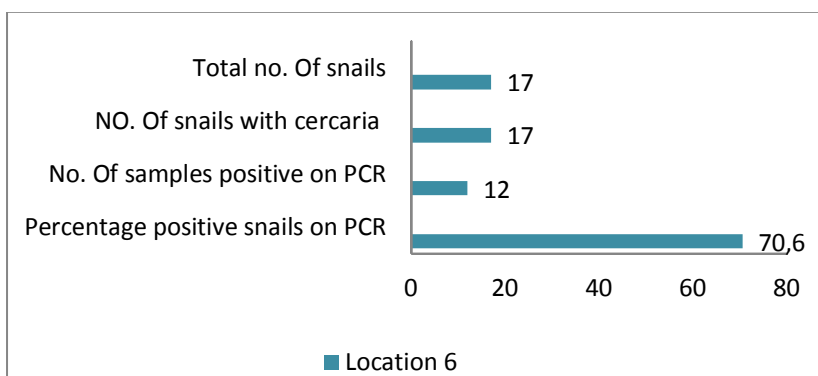


Fig. 10. Graphic representation of PCR results obtained from cercaria released from snails collected from Surducu Mic

No snails were identified in location 7 and 8 Săceni – Timiș and Vinga – Arad despite being known as areas infested with *Fasciola hepatica*. Thus, we cannot speak of cercaria.

Conclusions

One hundred and twenty three snails (51.3%) were positive for *F. hepatica* cercaria, confirmed through PCR.

The prevalence of parasitism with juvenile forms of *F. hepatica* varied according to the location from where the snails were collected from 40 to 70.6%. No snails were identified in two of these locations.

The present study brings forth a very important disease of economic interest animals and it is the first of its kind in Romania.

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OVERVIEW ON THE DIAGNOSIS AND TREATMENT OF CANINE ATOPIC DERMATITIS (CAD) -WHAT IS OLD AND WHAT IS NEW

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Summary

Canine atopic dermatitis (CAD) is a multifaceted disease, with causes among the most various-from environmental allergens to food allergens.

The diagnosis of this disease is very challenging for the veterinarian because none of the signs are pathognomonic. Various criteria regarding the clinical signs have been selected as eligible in order to help the clinician set a diagnosis. Despite the fact that serology is helpful in identifying the implicated allergens, it is of no use for the primary diagnosis of the condition.

The treatment options are also a challenge especially from the pet-owner-vet relationship point of view. Since CAD is a life-long condition, it is very difficult for the clinician to give the owner a treatment plan, which can satisfy him financially and to have a quick onset in relieving their pet from symptoms. The classical treatment includes corticotherapy and antihistaminic drugs. More recent treatment options are based on immunotherapy or immunomodulating agents such as oclacitinib (Apoquel), cyclosporine or tacrolimus and the latest discovery in the field of CAD treatment is based on monoclonal antibodies that target a key-point of the allergic disease: IL-31 (Cytopoint).

Keywords: Canine, atopic, dermatitis, treatment, diagnosis

Atopic dermatitis, in current understanding, is defined as a clinical disease associated with the presence of allergen specific IgE and with characteristic clinical signs (erythema, pruritus). In veterinary medicine, the term of atopic dermatitis (AD) is used to describe an environmental allergic skin disease (triggered by dust mites, pollens, etc.)

The disease management requires a multimodal approach in order to decrease the intense pruritus induced by the condition. AD is frequently accompanied by secondary infections or concurrent allergies leading to difficulties in the clinician's approach to pruritus minimization. The chronic character of AD is also an obstacle in the efficient treatment of AD, influenced especially by the owners' willingness to comply and understand the nature of the condition. Allergic diseases are currently diagnosed with an increased frequency both in animals and humans, probably due to the changes in the environment. While in humans, AD frequently progresses from the cutaneous form to the respiratory one, this process is rarely seen in the animal counterpart (20).

Clinically, the most important sign is pruritus, especially in the area of the feet, face and axillae, erythema, papular eruptions, self-induced alopecia, excoriations, hyperpigmentation, additional yeast or bacterial infections and some non-cutaneous

conditions associated with canine AD such as rhinitis (20-30% of cases) or conjunctivitis (7%) (22).

Diagnosis

Atopic dermatitis is defined as a genetically predisposed inflammatory and pruritic, allergic skin disease. However, it is very important to remember that this disease has no pathognomonic clinical signs thus, not permitting a definitive diagnosis upon initial consult.

Clinician Guidelines

The diagnosis of AD should involve three distinct approaches, namely:

1. Ruling out of other skin conditions with similar clinical signs- ectoparasites (*Sarcoptes*, fleas, demodicosis, cheyletiellosis, pediculosis, *Otodectes cynotis*, Trombiculiasis) microbial skin infections (Staphylococcal pyoderma, Malassezia dermatitis), other allergic skin diseases (flea allergy dermatitis, food allergy, insect bite hypersensitivity, contact dermatitis) and neoplastic diseases (cutaneous lymphoma) (22).
2. Detailed interpretation of the patient's history and a good-look at the clinical signs- which initially include scratching, pruritus, rubbing, chewing, excessive grooming or licking, head shaking (9). Depending on the involved allergens, the pruritus may be seasonal (pollen) or non-seasonal dust-mites, food). The representative areas affected in case of AD are the face, ear pinnae, ventrum, axillae, inguinal area, perineal area and distal extremities (17).
Another useful tool in the clinical diagnosis of AD are Favrot's criteria (Table. 1) (17)-a set of criteria developed from a complex statistical analysis of relevant clinical cases that lead to the creation of 2 sets of highly specific criteria (9).
3. Assessment of skin reactivity via IntraDermal Testing or detection of allergen-specific IgE (allergy testing)- the use of these methods is largely based on several factors such as severe and very frequent clinical signs, insufficient management with symptomatic therapy due to side effects of drugs or poor owner compliance (14). The tests however, are only recommended to identify the incriminated allergen when allergen-specific immunotherapy is required as treatment (15). This can be performed either by intradermal testing (IDT) or by allergen-specific IgE serology (ASIS) (18). The second technique has several advantages such as no need for sedation, less trauma for the patient (no need for repeated injections), less time-consuming, no need for clipping and the risk of interference with other drugs is very low (16). The disadvantage of this technique, compared to IDT is that it only measures circulating IgE and does not take into account other allergic pathways, often showing positive results in non-allergic dogs. On the other hand, studies have shown that 10-30% of clinically confirmed AD patients show negative IDT results (probably due to poor technique, low test concentration of allergens, drug interference, and incorrect selection of allergens or IDT performed during peak

allergy season or too long after it) (27, 33).

Applying these techniques individually can lead to a misdiagnosis and it is essential for the clinician to bear in mind that the diagnosis protocol should closely follow the up-mentioned steps.

Table 1

Favrot's sets of criteria for AD diagnosis (9)

	Use	Reliability
Set 1:	• Use for clinical studies and adapt required criteria based on the goal of the study.	• 5 criteria:
1. Age at onset <3 years	• If higher specificity is required, 6 criteria should be fulfilled (e.g., drug trials with potential side effects)	Sens. 85.4 %
2. Mostly indoor	• If higher sensitivity is required, 5 criteria should be fulfilled (e.g., epidemiological studies)	Spec. 79.1 %
3. Corticosteroid-responsive pruritus		
4. Chronic or recurrent yeast infections		• 6 criteria:
5. Affected front feet		Sens. 58.2 %
6. Affected ear pinnae		Spec. 88.5 %
7. Non-affected ear margins		
8. Non-affected dorso-lumbar area		
Set 2:	• Use to evaluate the probability of the diagnosis of canine AD	• 5 criteria:
1. Age at onset <3 years	• 5 criteria should be fulfilled	Sens. 77.2 %
2. Mostly indoor	• Do not use alone for diagnosis of canine AD, and rule out resembling diseases	Spec. 83 %
3. "Alesional" pruritus at onset		• 6 criteria:
4. Affected front feet		Sens. 42 %
5. Affected ear pinnae		Spec. 93.7 %
6. Non-affected ear margins		
7. Non-affected dorso-lumbar area		

Treatment

Type I hypersensitivity reactions are caused by the degranulation of mast cells so it should be obvious that **antihistamines** (20), which are the specific treatment against allergic inflammation, are a good choice for therapeutic management. However, their efficacy in the case of AD is limited because histamine in this case is not a major mediator for cutaneous inflammation and pruritus and antihistamines cannot act once histamine has already been bound to receptors. In conclusion, antihistamines can only be used as combination therapy in order to decrease the dose of other drugs, in long-term management of AD. One such combination strategy implies establishing the minimal dose of glucocorticoids that controls clinical signs and reducing it by 50% while adding in antihistaminic agents (hydroxyzine 2 mg/kg twice daily or cetirizine 1 mg/kg once daily) (5).

Glucocorticoids used in AD reduce production of inflammatory mediators and decrease the number of inflammatory cells-the two major influencers of allergic

response in case of AD. The advantages of glucocorticoids include low price and fast onset of action but the side-effects of these drugs (polydipsia, polyphagia, obesity, muscle atrophy, bacterial and fungal infections, demodicosis, skin atrophy, iatrogenic hyperadrenocorticism) are major disadvantages (28), making them a common indication for the control of acute flares or seasonal AD or even while other treatments are being implemented (during first weeks of immunotherapy or ciclosporin) (8). Use of topical glucocorticoids can be used to replace systemic therapy but only if the lesions are localized (26).

Ciclosporin, a calcineurin inhibitor, interferes in the synthesis of cytokines such as interleukin (IL)-2 and interferon (IFN)- γ - major components in the allergic reaction noticed in AD (30).

Ciclosporin is similarly effective with glucocorticoids but with considerably fewer and less severe side effects. The downsides are the higher cost and a slower onset of action which is the reason why this drug is only recommended for long-term treatment (25).

Tacrolimus, a topical calcineurin inhibitor, can be used to treat localized inflammation and pruritus but there are two major disadvantages to this product, namely: high cost and difficulty in applying the ointment as well as transient signs of topical irritation (32).

Oclacitinib, is a Janus kinase inhibitor that blocks signaling after binding of several pro-inflammatory and pruritus inducing cytokines to receptors (12). This novel anti-allergic component is very effective and with little side-effects (11), recommended for both acute flares and long-term management (usual dose is 0.4-0.6 mg/kg orally, twice daily and can be reduced to once daily after 2 weeks' time) (3, 19).

The compared efficacy (17) of the most common drugs or treatment modalities used in the management of AD is shown in Table 2.

Table 2

Some treatment modalities used in the management of AD (17)

TREATMENT	EFFICACY
<i>Antihistamines</i>	None to moderate
<i>Allergen specific immunotherapy</i>	Moderate to high
<i>Avoidance of environmental allergens</i>	Low
<i>Ciclosporin</i>	High
<i>Fatty acids (supplementes)</i>	Low
<i>Glucocorticoids (topical +systemical)</i>	High
<i>Oclacitinib</i>	High
<i>Tacrolimus</i>	High

Epidermal barrier dysfunction is a consistent aspect in dogs suffering from AD (2). **Fatty acids**, which have a role in the improvement of this defect, can and should be included in the therapeutic approach (1). The preferred fatty acids in order to achieve this goal are the n-6 fatty acids (e.g. linoleic acid) (29). Clinical efficacy can take several weeks to install, thus making oral supplementation with fatty acids an indication for long-term clinical management and only as an integrating part of a complete protocol (24). Recently, spot-on formulations containing fatty acids have also become available but they also should not be regarded as a possible unique treatment because their clinical benefit and role in reduction of pruritus is moderate and inconsistent (7, 31).

Current **ASIT** (6) in dogs is based on subcutaneous or sublingual administration of allergens (4) but a more recent approach, namely the intralymphatic route (10), has had good results in humans and studies have been extended on dogs as well. The most common allergen preparation used in therapy differ in USA-predominantly aqueous and Europe-predominantly allum-precipitated allergen extracts. The issues with this protocol and the reasons why it is still used by very few clinicians are the long period of time necessary for the treatment (up to 1 year) and the high costs involved as well as lack of standardized procedures and allergen extracts. New ideas however, are emerging in this filed, inspired by good results obtained in human medicine such as modified allergen preparations such as allergoids, recombinant major allergens, allergen peptides, use of adjuvants such as IL-10 inducers, packaging in virus-like particles or mucoadhesive polymers (6, 13).

Lokivetmab (Cytopoint™) is a caninized anti-canine-IL-31 mAb that specifically binds to circulating IL-31, inhibiting its binding to receptors (23). The neutralization of IL-31 resulted in pruritus decrease over a period of up to 8-weeks in dogs, following a single dose. Studies have shown that increased doses led to a better response (minimum effective dose 1 mg/kg s.c) and pruritus is reduced within one day, maintaining effectiveness up to 1 month (21). The fact that lokivetmab can be dosed in a single monthly injection can also be beneficial to the compliance of owners and it might allow a good monitoring of dogs with chronic AD (13).

Conclusions

There is no singular way of establishing a reliable diagnosis in case of AD, only a combination between history investigation, correlation with clinical signs and ruling out other dermatological issues which have similar symptoms.

Treatment options are under a continuous process of renewal and are constantly increasing in variety. Thus, clinicians have a wide range of substances or protocols, starting from the classical glucocorticoids, ciclosporin and antihistamines and ending with new trends in allergen-specific immunotherapy or different types of inhibitors such as Oclacitinib (Janus-kinase inhibitor) or lokivetmab (anti-canine IL-31).

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DETECTION OF POLYPARASITISM IN A LION HEAD RABBIT AND THERAPEUTIC PROTOCOL RESULTS. CASE REPORT

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Summary

In the Parasitology Clinic of the Faculty of Veterinary Medicine Timisoara, an owner brought a 7-month-old Lion Head rabbit. The reason for the consultation was the presence of auricular injuries. Skin dermatological examination, microscopic examination of the skin scrapings and fecal exams were performed. The results revealed the presence of *Psoroptes spp.* mites in the rabbit's ears, a large population of *Pulicidae spp.* fleas and *Eimeria spp.* oocysts. A topical acaricide and therapeutically protocol against protozoa has been applied. After 12 days of treatment, remission of auricular lesions, reduction in the number of oocysts and the flea population were observed. A rabbit polyparasitism can have serious health effects, but a suitable therapeutic protocol can heal the animal.

Keywords: rabbit, polyparasitism, treatment

Psoroptes cuniculi mites are the most common ear parasites infesting breeding female rabbits. The suffering rabbits show cutaneous signs of the infestation and are prone to secondary infections (4, 8). This species of mites can cause weight reduction, intense pruritus and crusting (7). It's importance resides in the zoonotic potential (1).

Pulicidae spp. flea species, through their feeding habits, can cause lesions on the skin that can even lead to depilation. *Spilopsyllus cuniculi* it is one of the most common flea found around Europe. It has a pronotal comb or a row of spines just behind the head and a general comb of spines below the head. These traits help to distinguish it from other species of fleas (3). These fleas can be transmitted to humans and cause pruritus and erythema, especially on the arms (6).

Coccidiosis is a major health problem in rabbits. It is caused by a protozoal parasite, represented by *Eimeria spp.* The oocysts are eliminated with the feces and will contaminate the environment, the food and water. This certain infection can also appear in well cared rabbits, especially in the youth (2, 5).

Materials and methods

In the process of diagnosis, we used several methods and exams. A general dermatological exam with careful examination of the entire body was performed.

Microscopic examination was performed on the scrapings and samples taken from the ears using cotton swabs.

A fecal exam using the Willis flotation method was executed in order to identify any internal parasites present.

A 1% Amitraz solution (Tactik) followed by 15 mg of spot-on product containing Selamectin (Stronghold puppy) was used for the treatment of mange mites and fleas. And for the coccidian infestation, a product based on Sulfquinoxaline (Sulfacoccirom) was administered in the drinking water.

Results and discussions

As a result of the exams and methods used, we discovered several elements of infestation.

A considerably large flea population that had led to skin lesions, discovered through the dermatological examination of the dermis (Fig. 1).

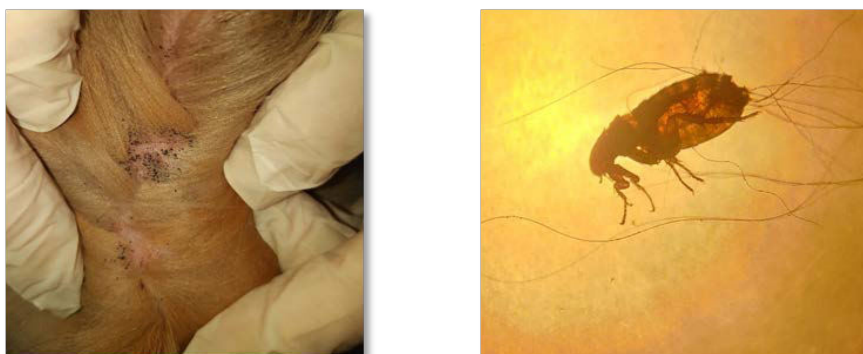


Fig. 1. The presence of *Spilopsyllus cuniculi* adults on the skin (left) and under the magnifying glass (right)

Mange mites (Fig. 2, 3) were discovered through the microscopic examination of the scrapings.

Eimeria oocysts were discovered through the Willis flotation method performed.

On the 31st of October, we were presented with a rabbit, which came to the clinic of the University because of auricular injuries. We had to determine the nature of it and decide on a course of action.

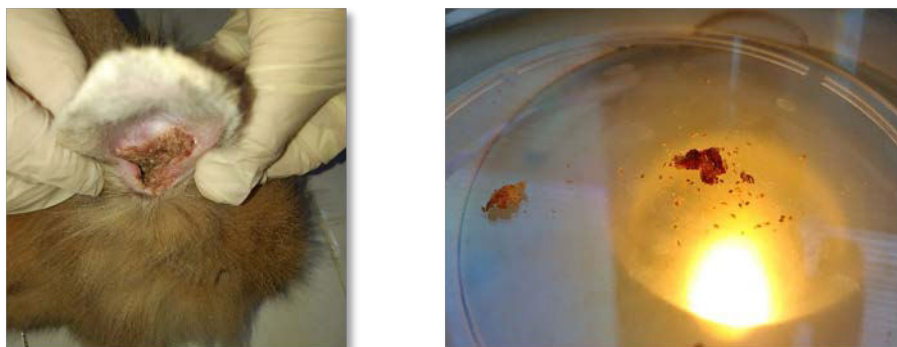


Fig. 2. Crusts and scabs in the ear (left) and the macroscopic aspect of the Petri plate (right)



Fig. 3. The aspect of the mange mites under the magnifying glass

We asked the owner about the rabbits' living conditions, discovering that the animal was acquired from a pet market in the city. The rabbit was living in an apartment, but also had access to the outside area surrounding the apartment building.

The decision to hospitalize the rabbit was made. He was held isolated from other animals to make sure the mange or flea infestation would not spread to the other animals in the hospital.

The ears were first cleaned mechanically using cotton swabs soaked in a solution of warm water and soap. The process was done thoroughly and targeted the removal of the debris, cerumen and loose crusts from the inside of the ears. This ensured a cleaner area on which we could start applying the treatment.

On following days, we repeated the process of cleaning his ears using the same solution and administered one drop of Tactik in each ear afterwards. Both ears were visibly cleaner after only two such processes.

We bathed him, on the second day of his hospitalization, using Tactik, a solution containing Amitraz in hopes of removing the massive flea population. After the bath we waited for his fur to dry, while keeping him under observation to make sure that no side effects occurred, considering his frail condition. After the bath, in which we specifically avoided the head area, the fleas started migrating forward, from the body to the head and gave us the clear response that the solution was effective in eradicating them (Fig. 4).



Fig. 4. The macroscopic aspect of the rabbit after the bath with Amitraz solution (left, middle) and the aspect of the cerumen under the magnifying glass with a reduction of the mite population

The team continued to clean his ears throughout the following days. The solution used for bathing started taking effect and we noticed a decrease in the number of fleas in general, but also, an increase of fleas on the head, especially around the eyes and between the ears.

On the sixth day, after cleaning his ears, we administered the spot-on product to ensure an even larger decrease in flea numbers and to make sure that the rabbit would be protected for a certain period.

We procured the anticoccidial treatment and started administering it into his drinking water. The substance was administered for two days, after which we paused the treatment the following two days. This period gave the treatment time to take full effect. After the rabbit's release from the hospital we instructed the owner on the administering anticoccidial treatment and advised him to continue with this treatment for the following week.

Within a couple of days after the treatment started taking effect we met with the owner and discussed the course of actions he had to follow to eliminate the fleas and the mites from his home which, from the state in which the rabbit was

presented, would be heavily infested as well. His home had to be thoroughly cleaned to avoid re-infestation of the rabbit, once he would be released from the hospital. We advised that these methods of cleaning be applied a few days before the release from the hospital, so that the solutions would have enough time to take effect and ensure a sterile environment for the rabbit, once he returned to his home.

Within a 12 days' time period the mites from the ears were completely gone and the lesions were starting to heal. The flea population decreased to near extinction and the Willis turned out negative on the last fecal exams. The rabbit was released from our hospital and was due for a reexamination the next week. The owner was given detailed instruction about the anticoccidial treatment administration and the future treatment for ectoparasites.

After one week (Fig. 5), the owner returned with the rabbit and we performed a complex examination, including a fecal exam. The auricular lesions were completely healed, there were no mites or fleas observed inside the ears and on the skin. The fecal exams results were negative after treatment.

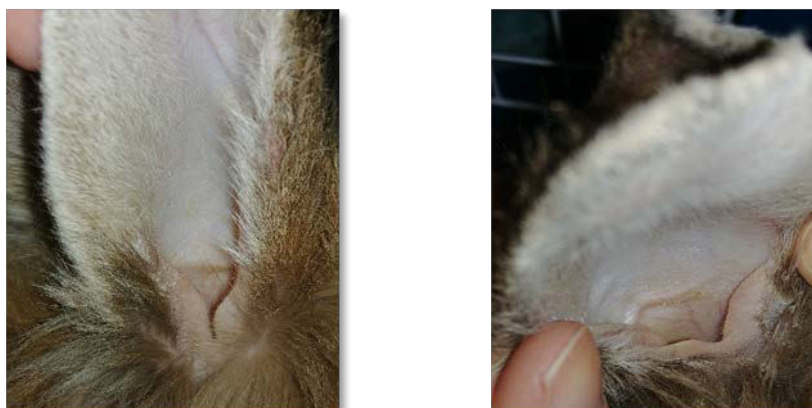


Fig. 5. The aspect of the ears after the final examination (right and left)

Conclusions

In case of *Psoroptes cuniculi* mites and *Pulicidae spp.* fleas the use of Amitraz solution and a topical product based on Selamectin led to remission of the auricular lesions and extinction of the flea population. The product based on Sulfaquinoxaline was efficient against coccidiosis.

A rabbit polyparasitism can have serious health effects, but a suitable therapeutically protocol can heal the animal.

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MONITORING THE INTESTINAL BARRIER FUNCTION USING TEER MEASUREMENT TECHNIQUE

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Summary

Over the time, *in vitro* models have been developed to study molecular transport across barrier tissues. The epithelial and endothelial cell layers provide a barrier between different tissues while also selectively transporting molecules across the barrier. The presence of tight junctions (*zonula occludens*), specifically regulate the flow of ions and solutes through the paracellular space, and the adherens junctions, regulate cell-cell interactions.

To study the function of a barrier tissue, especially an *in vitro* cultured barrier tissue, the cell layer must have high integrity.

This property can be measured using a simple and very convenient technique, the transepithelial/transendothelial electrical resistance (TEER).

The electrical resistance values measured by this method are a powerful indicator to assess the integrity of cell monolayers before they are used as experimental models to determine the transmembrane transport of some drugs or chemicals.

The equipment used for TEER measurements in epithelial cells in culture is the Millicell ERS-2 (Electrical Resistance System). TEER measurements can be performed in real time without cell damage. An increase in TEER detected with the electronic circuit of the Millicell ERS-2 meter and its electrode is an indicator of cell monolayer health and confluence.

Keywords: TEER, cultured cell, intestinal barrier

The purpose of this paper is to familiarize 2nd year veterinary students with modern techniques (TEER, respectively) and to further include those in the Animal Physiology practical work schedule.

The intestinal epithelium is a complex structure, with multiple functions resulted from the capacity of barrier and selective transport of the cell.

TEER is the acronym of transepithelial electrical resistance. TEER is a quantitative method that evaluates the integrity of tight junctions from cell cultures.

The tight junctions (Fig. 1) are those structures between cells that prevent uncontrolled passage of substances through the paracellular pathway. Also, tight junctions create selective channels for the paracellular transport of cations, anions and water.

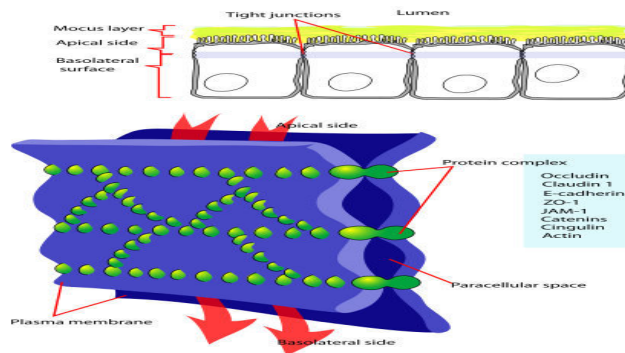


Fig. 1. The diagram of a tight junction (15)

Materials and methods

A. The TEER measurement components

The equipment (Fig. 2) required to perform TEER measurements is: volt-ohm meter, electrode and cells cultured on specific inserts with permeable support (16, 17, 18).



Fig. 2. TEER measurement components

B. The principle of TEER technique

The first electrode emits an electric current that passes through the layer of cells and the semipermeable membrane, while the second electrode measures the current on the other side of the membrane (Fig. 3). The resistance of the cell layer is proportional to the number of tight junctions developed (12, 16, 17, 18).

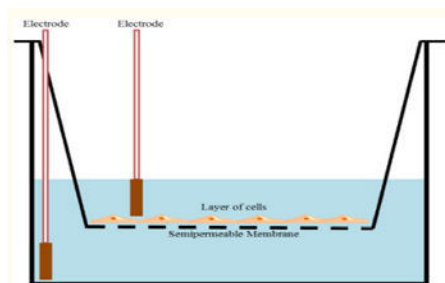


Fig. 3. The principle of TEER technique (12)

C. Gastrointestinal tract models

Nowadays, there are numerous cell lines studied for *in vitro* models of the gastro-intestinal tract. One of the most used cell line who re-create gastrointestinal tract *in vitro* models is the Caco-2 line. These cells are maintained in cell culture for many weeks and they can establish tight junctions in the culture. After 2-3 weeks, Caco-2 line form a densely populated cell layer differentiating spontaneously into polarized enterocytes and a monolayer that are combined by tight junction protein complexes.(2, 6). The Caco-2 monolayer generates a TEER of 150-400 Ω/cm^2 that restricts the diffusion of substances across the barrier.

Compared to Caco-2 cell lines, HT29-MTX lines will produce different barrier properties when they are introduced into the culture. To prevent alteration of barrier functions it is recommended to test the permeability of cultured cells using TEER method (5).

Another popular cell model is the IEC-18 cell line. This cell line is composed of cells obtained from rat intestine. A study realized by Steensma et al. (13), showed that these cells can simulate the physiological properties of the small intestine. However, this cell line is less well differentiated than the Caco-2 cells and not much is known about the carrier-mediated transport systems.

The latest cell model we are talking about in this review is the TC-7 cell model. These cells are subclones of the Caco-2 cell line but they have a higher amount of enzymes compared to the Caco-2 model, as shown in a study made by Gres et al. in 1998 (3).

Results and discussions

Odiijk M, et al. (8) showed that the TEER measurements are influenced by: the medium interface, the temperature, the supporting material for the cell layer, the electrode and the membrane area.

Being a modern, accurate and easy-to-use method, TEER technique has an extensive applicability in medicine and biology fields like physiology, cell biology, pharmacology, nutrition and toxicology.

In physiology, using the TEER technique, it was possible to distinguish between types of epithelium by tight junction presence (1), obtaining three main classes: tight epithelia, intermediate epithelia, leaky epithelia.

- Tight epithelia: this epithelium has many tight junctions between its cells and that makes it impermeable for most molecules. Using TEER method, high values of the electrical resistance are measured (2000 Ω/cm^2). The gastric epithelium is a good example of tight epithelia.
- Intermediate epithelia: this epithelium is impermeable for some molecules, but permeable for others. Using TEER method, the measurements will reveal medium values of the electrical resistance (200 Ω/cm^2). The epithelium of the colon is an example for this kind of epithelia.
- Leaky epithelia: this epithelium has a low number of tight junctions between its cells and that makes it very permeable for most molecules. Using TEER method, low values of the electrical resistance (50-100 Ω/cm^2) are specific for the small intestine epithelium, the main site of absorption.

In pathophysiology some studies involving ischemic injuries of piglets intestinal mucosa revealed that injuries caused animal death if the mucosal barrier function wasn't restored fast. Young and his collaborators (14) realized a study on 6-8 weeks old piglets and noticed that neonatal necrotizing enterocolitis (NEC) is characteristic for intestinal ischemia.

Other studies made by Messineo (7), Lee (4) and Seashore (11) have shown that the probability of intestinal ischemia is higher for newborn piglets up to 1-2 weeks. These studies showed a fast repair of ischemia-injured mucosa for 6-8 weeks-old piglets, but in case of new born piglets (2 weeks-old), the mucosa failed to recover and the mortality percentage was 100%.

In pharmacology, Sarmiento et al. (10) studied the effects of the drugs on the intestinal mucosa using TEER method. The transport of drugs through the intestinal epithelia is a complex process that includes the passage of compounds across several pathways. To complete their study, they used a cell-line cultured *in vitro*. They observed that the properties of the intestinal mucosa are modified: for example, the transelectrical resistance of the small intestine increased from 100 Ω/cm^2 to 180 Ω/cm^2 .

In 2015, using TEER measurements, Placha and al. (9) observed the effect of *Salvia officinalis* essential oils (EO) on the permeability of the duodenal epithelium. By adding 0.1g *Salvia officinalis* EO/kg on the diet of chickens, Placha observed that transepithelial electrical resistance increase in duodenal tissue compared to transepithelial electrical resistance values measured in chickens who ate a normal diet, without *Salvia officinalis* EO.

Conclusions

TEER measurement technique is a modern, accurate and easy-to-use method with extensive applicability in research, biology and medicine. Using this method, animals don't have to be sacrificed in order to obtain cell cultures.

TEER measurement is a valuable non-invasive technique that can be applied to quantify the barrier integrity of cells during their various stages of growth and differentiation.

Numerous *in vitro* cell models composed of immortalized cells that can be grown on semipermeable supports under controlled conditions have been studied for drug permeability and absorption evaluation. The success of these studies to predict drug absorption depends on how closely the *in vitro* models can mimic the complexity of the drug absorption processes of the *in vivo* barriers. By measuring the electrical resistance using the TEER technique, we can evaluate the integrity of the neoeepithelium and the barrier function of the intestinal epithelium.

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PREVALENCE OF OVINE (*OVIS ARIES*) NON-NEOPLASTIC PULMONARY LESIONS IN TRANSYLVANIA (ROMANIA)

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Summary

Ovine respiratory diseases represent a major problem among sheep populations due to significant economic losses, especially affecting the adult animals. The purpose of this study was to determine the prevalence of the main ovine respiratory diseases in some counties from Transylvania (Romania); a detailed post-mortem characterization of the non-neoplastic pulmonary diseases of sheep was also performed. The samples were represented by pulmonary tissues that showed macroscopic changes, collected as a result of examination of 2349 lungs in two major slaughterhouses from Transylvania region (Sibiu and Bistrița Năsăud counties). Samples were collected and processed appropriately for histological, microbiological and parasitological examinations. In this study, a very high prevalence (82.67%) of lung parasitic diseases was noticed. The highest prevalence was recorded for verminous pneumonia (58.1%), followed by mixed parasitic infestation of *Strongylus spp.* and *Echinococcus spp.* (14.9%). The predominant parasitic disease was the co-infestation with *Protostrongylus spp.* and *Muellerius spp.* Bacterial pneumonia with gross features consisting with caseous lymphadenitis and pasteurellosis had a relatively low prevalence (2.8%). Non-tumoral viral infections with lentivirus (ovine progressive pneumonia-Maedi) was encountered in 0.97% of the cases. No pulmonary lesions were detected in 13.56%. We concluded that parasitic infestation is the most important pulmonary disease in sheepflocks in Transylvania. Similar studies covering other geographical areas from Romania in order to obtain a complete picture of the ovine pulmonary pathology in our country are needed.

Keywords: epidemiology, sheep, pneumonia, strongyloses

Ovine respiratory diseases result in loss of body condition, decrease of fertility and high mortality, with considerable financial losses for breeders.

Various factors, including abnormal weather changes, physiologic stress, and different infections with viruses (e.g. Parainfluenza virus type 3) predispose for development of severe pulmonary bacterial diseases (7). Age of the animals, the type of nutrition and the geographic area where sheep live are considered determining factors for different types of etiological agents (3).

Manheimia haemolytica, *Pasteurella multocida*, *Histophilus somni*, haemolytic streptococci, *Biberstina trehalosi*, *Helococcus ovis*, *Mycoplasma ovipneumoniae* and *Corynebacterium pseudotuberculosis* are the main bacterial agents which affect both young and adult animals (5). Lung abscesses are common in adult sheep (17) and *Trueperella pyogenes* is the most common bacteria isolated from these lesions (6).

Verminous pneumonia is reported worldwide with a rate of occurrence 3.8 – 4.7% in small ruminant population with *Dictyocaulus filaria* (12) and *Muellerius capillaris* (5) reported as the most common lungworms. Other pulmonary parasites that can be encountered in sheep and goats are *Protostrongylus rufescens*, *Cystocaulus ocreatus* and *Neostrongylus linearis* (5).

Romania is a well-known country for sheep farming with two main autochthonous breeds: Țurcană and Țigaie; the first sheep breed represents 52.5% of the national breed herds (11).

The aim of this study was to evaluate the epidemiological status of the non-neoplastic pulmonary lesions in autochthonous sheep breed (Țurcană) from Transylvania (Romania). A detailed pathological description of the most frequently encountered diseases was also performed.

Materials and methods

The study was performed in two slaughterhouses from Transylvania area (from Sibiu and Bistrița-Năsăud counties) in the period between November 2017 and October 2018. A number of 2349 lungs were grossly evaluated and all changes were noticed in a necropsy report of an individual animal.

For histological examination, the samples were fixed in 10% formalin for 48-72 hours followed by routinely embedded in paraffin. The sections (4-5 μm) were stained with haematoxylin-eosin (H&E). The histological examination was performed in the Department of Pathology, Faculty of Veterinary Medicine (Cluj-Napoca).

For parasitological examination the sample tissues were refrigerated (4-6°C) for maximum 12 hours until the samples were processed for direct examination and Baermann methods. The examination of the morphological characteristics of the parasitic organisms was performed by adding a few drops of formalin in order to immobilize the larvae. The parasitological exams were made in the Department of Parasitology, Faculty of Veterinary Medicine (Cluj-Napoca).

Results and discussions

The predominant non-neoplastic lesions encountered in our study were represented by pulmonary parasitic diseases (82.67%). The main parasitic diseases that we have encountered were represented by strongyloses, followed by mixed parasitic infestations (strongyloses and echinococcosis) and echinococcosis (Fig. 1). Parasitic pulmonary diseases showed different degrees of severity. A relatively small percentage of the evaluated cases were induced by infectious agents, including bacterial infections (2.8%) and viruses, such as Maedi-Visna virus (0.97%); 13.56% of the evaluated cases showed no gross lesions.

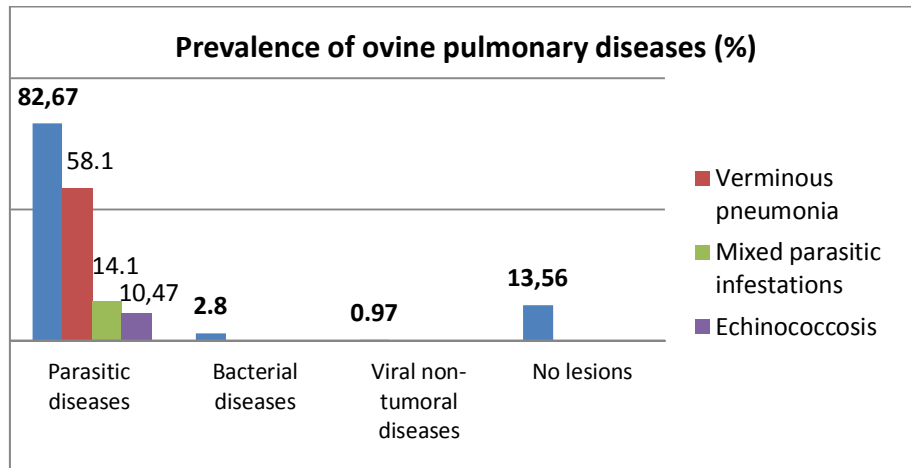


Fig. 1. Classification of the main pulmonary diseases in sheep from Transylvania (Romania): a high prevalence of parasitic diseases (82.67%) was encountered, in contrast with a low number of cases without any pulmonary disease (13.56%)

Eighty-five tissue samples were collected for parasitological examination in order to establish the species of parasites. The results showed that *Muellerius capillaris* and *Protostrongylus rufescens* were identified in 49 samples (57.64%), hydatid cysts in 13 samples (15.3%), and mixed infestations (hydatid cyst and strongyles) in 3 samples (3.53%); 20 samples were negative (23.53%) (Fig. 2).

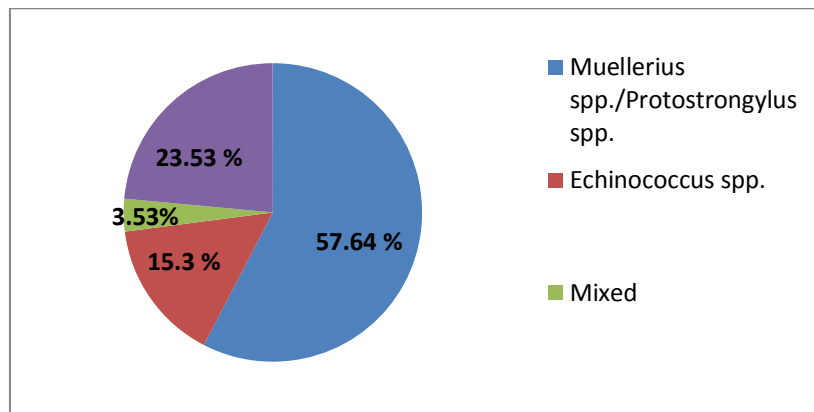


Fig. 2. The main species of parasites involved in ovine pulmonary diseases. Strongyloses caused by *Muellerius spp.* and *Protostrongylus spp.* were the main pulmonary parasites identified in the evaluated samples

Echinococcus spp. infestation was associated with numerous randomly distributed, large (5-8 cm in diameter) granulomatous nodules (Fig. 3A).

In verminous pneumonia the distribution of the pulmonary lesions has been related to the degree of infestation. Lesions of interstitial pneumonia associated to *Protostrongylus spp.* were usually found in the caudal lobes, as firm grey-brownish elevated plaques or nodules, that interested mainly the dorsal area of the pulmonary lobes; the lesions were occasionally surrounded by atelectatic or emphysematous areas (Fig. 3B, 3C).

In the severe cases of verminous pneumonia, the lesions occupied up to 80% of the pulmonary parenchyma and numerous adult worms were identified in the main bronchi (Fig. 3D). *Muellerius spp.* infestation produced multiple subpleural, small and firm pulmonary nodules (2-4 mm in diameter), mainly in the caudal lobes.

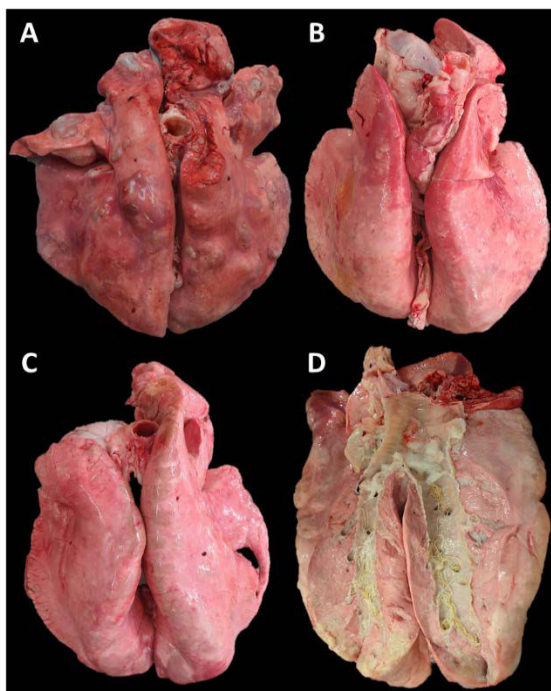


Fig. 3. Gross features of pulmonary parasitic diseases: A) Echinococcosis and mild verminous pneumonia; B) and C) Severe verminous pneumonia; D) Catarrhal tracheobronchitis with severe bronchial infestation with adult forms of strongyles

Bacterial pneumonia was identified mainly as randomly distributed pulmonary abscesses with a central area of necrosis; the necrotic material was

arranged in multiple concentric layers and enclosed by a fibrous capsule (Fig. 4B). *Corynebacterium pseudotuberculosis* infection was the main agent identified in these solitary lesions by bacteriological exam. In other cases, the abscesses contained a creamy yellow material and were disposed in the cranio-ventral area of the lungs, these lesions being compatible with chronic complications of manheimiosis (Fig. 4A).

Viral non-tumoral pulmonary diseases showed diffuse chronic interstitial pneumonia (Fig. 4C), with small gray foci (1-2 mm) on the cross surface (Fig. 4D) and enlargement of the trachobronchial and mediastinal lymph nodes. The lungs failed to collapse, they were heavy, weighing 2-3 more times as the normal weight. Viral non-tumoral pulmonary diseases were identified only in a small percentage of the evaluated cases and diagnosed as ovine progressive pneumonia (Maedi).

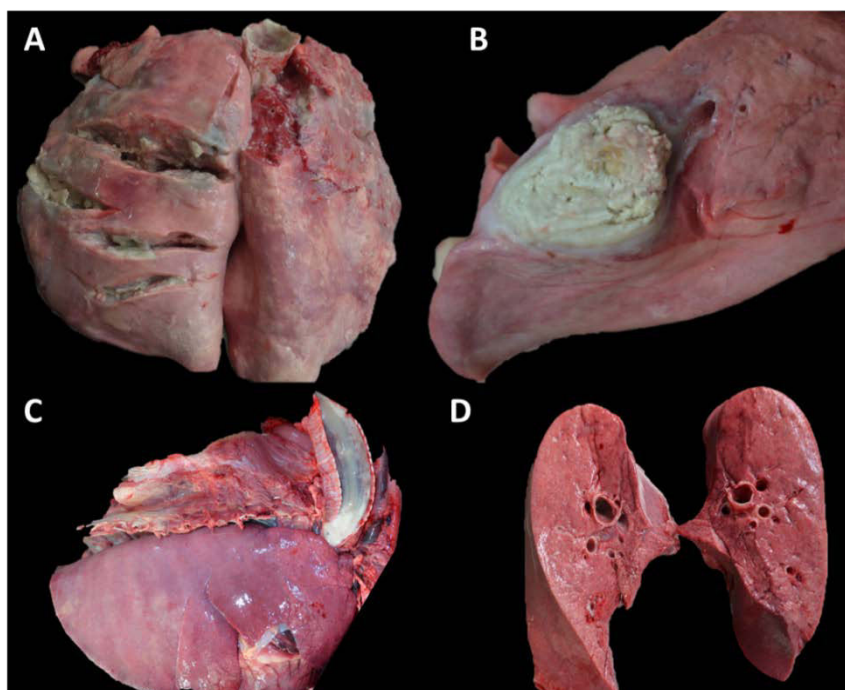


Fig. 4. Gross features of bacterial and viral pneumonias: A) Purulent bronchopneumonia (complication of Mannheimiosis); B) Pulmonary abscess produced by *Corynebacterium pseudotuberculosis*; C) and D) Diffuse interstitial pneumonia (Maedi)

Histological examination of lungs affected by parasitic diseases revealed 2 main types of lesions in accordance with the etiological agent: interstitial non-granulomatous pneumonia and granulomatous pneumonia.

Inflammatory changes attributed to *Muellerius spp.* infestation consisted of granulomatous reaction centered on parasitic organisms (adult forms, larvae and embryonated eggs). The granulomas are composed of a central area of necrosis and mineralization, and surrounded by macrophages, multinucleated giant cells, eosinophils, plasma cells and lymphocytes. The inflammatory reaction is enclosed by a thick fibrovascular capsule (Fig. 5A).

In the cases associated with *Protostrongylus spp.* infestation, the histological characteristics were diffuse interstitial pneumonia with presence of numerous larvae in the interstitium surrounded by large numbers of macrophages, lymphocytes, plasma cells and eosinophils. Interstitial edema, marked hyperplasia of the lymphoid tissue (BALT hyperplasia), smooth muscle cell hyperplasia and interstitial fibrosis were also observed in the affected areas (Fig. 5B).

In pulmonary echinococcosis the histological features are represented by a nodular granulomatous reaction centred on the parasitic organism. The hydatid cyst consists of a thick eosinophilic germinal layer, enclosing a parasitic cavity. The granulomatous inflammatory reaction is composed of numerous macrophages, macrophagic epithelioid cells, multinucleated giant cells, eosinophils and lymphocytes. Granulomas are delimited by a prominent fibrous capsule that separates the parasitic granuloma and normal parenchyma (Fig. 5C).

Infectious bacterial and viral pneumonia showed features of fibrinous and fibrinopurulent bronchopneumonia, pulmonary abscesses and interstitial pneumonia. In fibrinous bronchopneumonia, the alveoli are distended and filled with large amount of acidophilic substance (fibrin) admixed with foamy macrophages, lymphocytes and neutrophils (Fig. 5D). The alveolar septa are diffusely thickened due to the presence of inflammatory cells. Multifocal, type II pneumocyte hyperplasia was also observed. The bronchiolar epithelium was diffusely affected by necrosis and erosions, and the bronchial lumen was filled with large amount of fibrinous exudate. Multifocal, the pulmonary parenchyma showed areas of coagulative necrosis surrounded by a thick rim of neutrophils and macrophages (Fig. 5E).

Histologically, in ovine progressive pneumonia, the interstitial space was diffusely expanded by large numbers of lymphocytes, macrophages, plasma cells and fibrous tissue; hyperplasia of smooth muscle cells and lymphoid tissue (BALT hyperplasia) was also observed at this level (Fig. 5F). The alveoli were distended by edema, degenerated and sloughed pneumocytes admixed with macrophages and neutrophils. Although most of the studies (1, 2, 14), showed that the prevalence of parasitic infestations is higher in young animals (less than 1-year-old), in our study the age of the animals ranged between 2 and 4 years. Borji *et al.* (2012) obtained a prevalence of 4.1% pulmonary strongylosis by both post-mortem and parasitological examinations. In India, Mishra *et al.*, 2018 obtained a rate of

occurrence of 1.57% in verminous pneumonia. These results are significantly lower compared to our results, in which 82.67% of the examined lungs showed evidence of parasitic lesions. This evidence suggests that pulmonary parasitic diseases represents a major problem among Romanian sheepflocks. The explanation for this significant discrepancy can be related to an inefficient deworming treatment or even absent, but unfortunately this information was not available for our study. Also, the differences can be associated with nutrition, immune status, environment, humidity, temperature and altitudes from Romania. Even though the strongyles have a world-wide distribution, the highest infestation rate is encountered in the countries with a tropical and subtropical climate (1, 2, 10, 16).

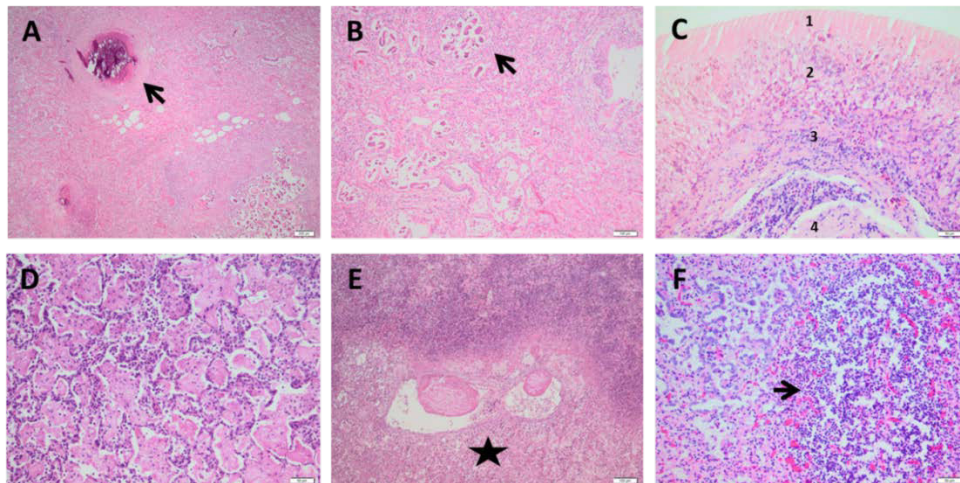


Fig. 5. Histological features of parasitic and bacterial pneumonia: A) Parasitic granuloma caused by *Mullerius spp.*. The granulomatous reaction showing a prominent central area of mineralization (arrow); B) Interstitial pneumonia with presence of aggregates of parasitic larvae of *Protostrongylus spp.* (arrow) in the interstitial space; C) Granulomatous pneumonia caused by *Echinococcus spp.*: 1- germinal layer, 2- epithelioid and parasitic multinucleated giant cells layer, 3 - eosinophils, lymphocytes and plasma cells, 4- fibrous capsule; D) Fibrinous bronchopneumonia caused by *Mannheimia spp.* infection; E) Necrotizing pneumonia characterized by large areas of necrosis (star) delimited by inflammatory cells; F) Viral interstitial pneumonia with lymphofollicular hyperplasia (arrow) (Ovine progressive pneumonia or Maedi). H&E stain

The low prevalence of pulmonary parasitic infestations in adult sheep is considered to be related to a stronger immune status and development of specific immunity (18). Regarding the infestation with *Dictyocaulus spp.* in sheep, our results showed that this type of pulmonary strongyle doesn't represent a major

problem among sheepflocks in Transylvania. The results are in accordance with other previous studies that showed similar incidence of *Dyctiocaulus spp.* in sheep (2, 16). In our study the predominant infestation was represented by association of *Protostrongylus spp.* and *Muellerius spp.* in 58% of cases.

The pulmonary lesions caused by lungworm infestation were identified mainly in the dorsal area of the caudal lobes, but in the severe cases the lesions also involved the cranial and middle lobes. In contrast to Panayotova-Pencheva and Alexandrov (2010), the lesions caused by *Mullerius capillaris* infection were nodular and only in severe cases or associated with *Protostrongylus spp.* infestation, the inflammatory processes affected large areas of pulmonary parenchyma.

In our study, the features of bacterial pneumonia consisted with ovine caseous lymphadenitis produced by *Corynebacterium pseudotuberculosis* and acute or complicated forms of *Mannheimia haemolytica* infection. Various agents, including *Actinobacillus licheniformis*, *Trueperella pyogenes* or *Staphylococcus aureus* subsp. *anaerobius* may also cause suppurative lymphadenitis (4, 15). The lesions consistent with mannheimiosis had a low prevalence. Bacteria responsible of epizooty in sheep are *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida* and *Mycoplasma ovipneumoniae* (8). In all cases, the differential diagnosis between different bacterial infections requires a bacteriological exam.

Conclusions

Pulmonary parasitic diseases represent a major problem in romanian sheepflocks. Most of the infestations were extremely severe, affecting both the welfare of the animals and their production.

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EFFECTS OF INTRAVENOUS GADOLINIUM ADMINISTRATION UPON PULSE RATE, RESPIRATORY RATE AND MEAN ARTERIAL PRESSURE ON GERIATRIC DOGS DURING MAGNETIC RESONANCE IMAGING

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Summary

This study was performed in order to evaluate the effects of contrast media administration upon pulse rate (PR), respiratory rate (RR) and mean arterial pressure (MAP) on geriatric dogs during magnetic resonance imaging (MRI). The study was conducted on 21 geriatric dogs that were presented at Faculty of Veterinary Medicine in Bucharest for neurological examination followed by MRI. Preanaesthetic examination was performed and patients assigned to their ASA II-III status (American Society of Anesthesiology). The patients were premedicated with Midazolam 0.2 mg/kg, Butorphanol 0.3 mg/kg and Ketamine 5 mg/kg intramuscularly (IM), induction was made with Propofol 4-6 mg/kg intravenously (IV). Afterwards, all patients were intubated and maintained with Isoflurane in 100% Oxygen. Gadolinium was administered 50 minutes after induction. Pulse rate, respiratory rate and mean arterial pressure were measured before intravenous administration of the gadolinium and 5 minutes after IV administration. The results suggested that minor variations of PR, RR and MAP were most frequent (12 dogs – 57,14%), followed by 3 dogs (14.28%) that had moderate variations and 6 dogs (28.57%) that didn't show any changes. Before administration of contrast media, the mean average results for MAP was 75.33 mm/Hg, for RR was 12.28 bpm and for the PR was 98,95 bpm. Five minutes after administration of the contrast media, the mean average results were for MAP 66.71 mm/Hg, for the PR was 96 bpm and for RR was 12.94 bpm. There is a significant positive relationship between the values of MAP, PR and RR before administration of the contrast media and 5 minutes post administration ($p < 0.05$). In conclusion, minor to moderate reactions to the contrast media are frequent; severe reactions represented by a decrease of the PR, RR or MAP are not likely to occur following administration of the contrast media for MRI procedure.

Keywords: contrast media, geriatric dogs, magnetic resonance imaging, reactions

Magnetic resonance imaging (MRI) is a diagnostic imaging modality that is widely used in veterinary medicine, in which a contrast study is often required for diagnosis. Contrast media administration can cause adverse effects upon pulse rate (PR), respiratory rate (RR) and mean arterial pressure (MAP) that can be classified as mild, moderate or severe (6, 9).

Gadolinium - based contrast agents (GBCAs) is the most commonly used contrast in MRI.

Gadobutrol is a second generation, extracellular, non-ionic macrocyclic GBCA that is used in patients undergoing MRI for visualization of pathological regions (8). The use of gadolinium based contrast agents is considered relatively

safe but adverse effects have been reported in both human (5, 4) and veterinary medicine (3).

In human medicine, reactions to contrast media have been extensively investigated; compared with the veterinary medicine where we talk about few studies, all of which have included small numbers of animals (2).

The aim of the present study was to investigate the effects of intravenous gadolinium administration upon pulse rate, respiratory rate and mean arterial pressure on geriatric dogs during magnetic resonance imaging.

Materials and methods

This article represents a clinical study conducted on 21 canine geriatric patients of different ages, belonging to different breeds that were anaesthetized for magnetic resonance imaging diagnosis. The study was conducted between September 2018 and November 2018 in the Faculty of Veterinary Medicine Bucharest on anaesthetized dogs that received intravenous (IV) gadobutrol for MRI. Dogs included in the study had records information on heart rate (HR), respiratory rate (RR) and mean arterial pressure (MAP) measured at 5 minutes before (baseline) and 5 minutes after contrast media administration. Gadolinium – containing contrast media was administered at doses of 0.2 ml/kg gadobutrol.

Preanaesthetic examination was performed and patients assigned to their ASA II-III status (American Society of Anesthesiology). All dogs were premedicated with Midazolam 0.2 mg/kg, Butorphanol 0.3 mg/kg and Ketamine 5 mg/kg intramuscularly (IM), induction was made with Propofol 4-6 mg/kg intravenously (IV). Afterwards, all patients were intubated and maintained with Isoflurane in 100% Oxygen. Intermittent positive-pressure ventilation (IPPV) was initiated by use of a volume-cycled ventilator. Oxygen flow was initially delivered at 2L/min, 2.0% Isoflurane within 3-5 minutes of induction. After 3-5 minutes, oxygen flow was decreased to $(500+10 \cdot \text{kg})$ L/min and Isoflurane maintained to a mean MAC of 1.7. Lactated Ringer solution was administered IV at 3-5 ml/kg/h throughout anaesthesia.

For each patient we recorded: age, breed, weight, pulse rate, respiratory rate and mean arterial pressure. In all MRI cases, pulse rate was obtained using a pulse oximeter. Because electrocardiography (EKG) was unavailable in MRI, respiratory rate was obtained using a capnograph and mean arterial pressure was measured non-invasively with an oscillometric method (Fig.1).



Fig. 1. Capnography and mean arterial pressure

Pulse rate, respiratory rate and mean arterial pressure were measured before intravenous administration of the gadolinium and 5 minutes after IV administration (Fig. 2).



Fig. 2. Pulse rate before and 5 minutes after gadolinium administration

Results and discussions

The results suggested that minor variations of PR, RR and MAP were most frequent (12 dogs – 57.14%), followed by 3 dogs (14.28%) that had moderate variations and 6 dogs (28.57%) that didn't show any changes. Before administration of contrast media, the mean average results for MAP was 75.33 mm/Hg, for RR was 12.28 bpm and for the PR was 98.95 bpm. Five minutes after administration of the contrast media, the mean average results were for MAP 66.71 mm/Hg, for the PR was 96 bpm and for RR was 12.94 bpm.

There is a significant positive relationship between the values of MAP, PR and RR before administration of the contrast media and 5 minutes post administration ($p < 0.05$) (Fig.3-5).

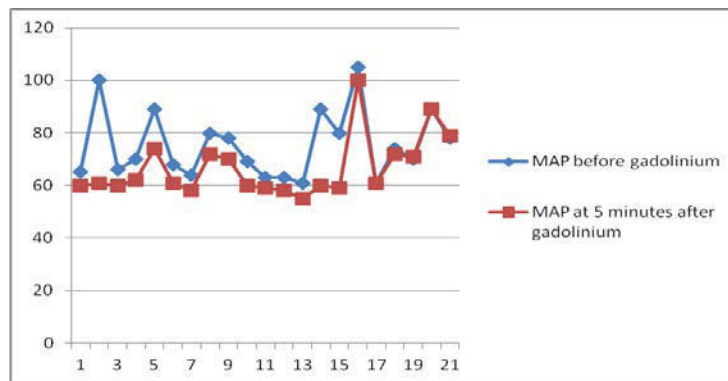


Fig. 3. Mean arterial pressure before and 5 minutes after gadolinium administration

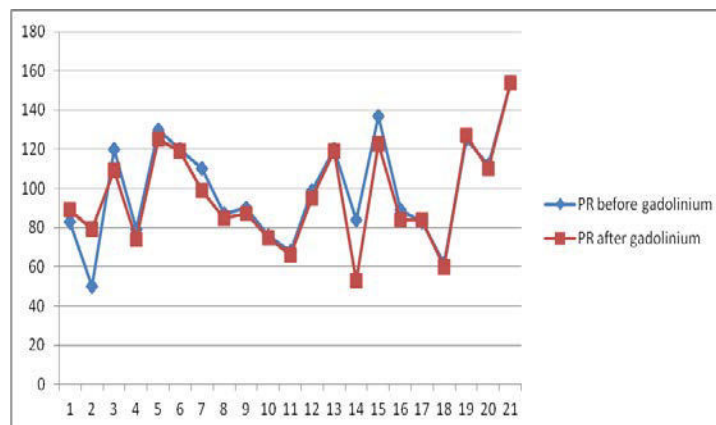


Fig. 4. Pulse rate before and 5 minutes after gadolinium administration

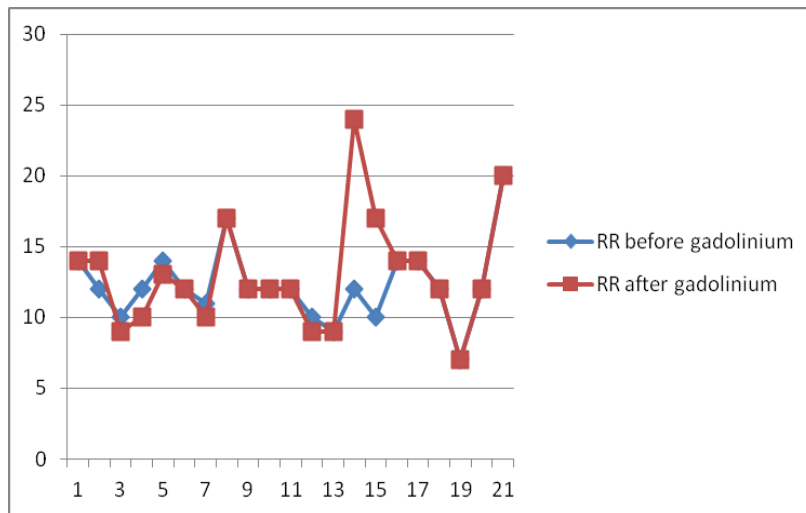


Fig. 5. Respiratory rate before and 5 minutes after gadolinium administration

In our patients, all the parameters measured changed in both directions, but changes in MAP from baseline always reflected a decreased compared with RR where 4 dogs had an increased measurement.

In human medicine, adverse effects to contrast media are uncommon; after GBCA administration the incidence of acute adverse reactions varies between 0.07% and 2.4% (5) and the most common reactions are mild to moderate. The incidence of severe life-threatening reactions after the administration of gadolinium – containing contrast media in humans is very low (0.001-0.01%) (5), although fatal reactions have been reported (6). As well as in humans, anaphylactoid reactions are reported in dogs (1, 3). In the present study, no patients had life-threatening reactions following administration of the gadolinium.

No drugs were administered or fluid rate changed within 5 minutes of or during administration of the contrast media, so any haemodynamic reactions observed were attributed to the contrast agent.

A predisposing factor to hypersensitivity reactions to gadolinium - containing contrast media in people is the previous administration of a contrast agent. Jung et al. reported that the incidence of hypersensitivity reactions increased according to the number of previous exposures to GBCAs from 0.105% after the first exposures to 0.171% after three or more exposures. In our study, no dogs had any previous exposure to gadolinium – containing contrast media.

Conclusions

In conclusion, minor to moderate reactions to the contrast media are frequent; severe reactions represented by a decrease of the PR, RR or MAP are not likely to occur following administration of the contrast media for MRI procedure.

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