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TIMIŞOARA**

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LUCRĂRI ŞTIINŢIFICE

**MEDICINĂ VETERINARĂ
TIMIŞOARA
VOLUMUL LII (2)**

**SCIENTIFICAL PAPERS
VETERINARY MEDICINE**

To be cited: LUCRARI STIINTIFICE: MEDICINA VETERINARA TIMISOARA (SCIENTIFICAL PAPERS: VETERINARY MEDICINE TIMISOARA)**,** vol. LII (2), 2019

Available online at: **http://www.usab-tm.ro/USAMVBT_Revista_ro_1086.html**
Indexed and/or abstracted in**:** CABI Full Text, CAB Abstracts, Ulrich's Periodicals Directory
Editor: AGROPRINT TIMISOARA ISSN: 1221-5295
Printed by: IMPRIMERIA MIRTON TIMISOARA

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**TIMIŞOARA
2019**

ESTRUS SYNCHRONISATION AND ARTIFICIAL INSEMINATION IN LACAUNE SHEEP

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Summary

The purpose of this research was to perform estrus synchronization and artificial insemination in sheep during the breeding season with progesterone (Chronogest, Maravet) and PMSG (Folligon, Maravet). The study was carried out during September 2018 - February 2019. The hormonal protocol was applied on 125 animals. After treatment ewes were artificial inseminated by intracervical method. The diagnostic of gestation was performed at 45 days after the artificial insemination using an ultrasound. The rate of fertilization, the prolificacy and the specificity of the ultrasound diagnosis were established after parturition.

Keywords: ewes, fertility, prolificacy, synchronization

Estrus synchronization is an important tool in a sheep reproduction management. During the past three decades, considerable efforts were made to develop methods for estrus synchronization in small ruminants to be used as a management tool to farmers and research studies. The key element of such methods is to control luteolysis and the corpus luteum lifespan. Improved pregnancy rates were reported in cattle receiving GnRH–$PGF_2\alpha$ protocol for estrus synchronization when progesterone was applied during treatment (5). The Rebound reflex is maybe the most utile method for made the estrus synchronization at sheep. Two types of sponges are currently commercially available, based on either flurogestone acetate (FGA), marketed as Chronogest (Intervet, Angers, France), or medroxyprogesterone acetate (MAP), marketed as Veramix (Pharmacia & Upjohn, Orangeville, Canada) (16). For a period of 10-16 days, the progesterone sponges has been successfully used for estrus synchronization in sheep during the breeding and the nonbreeding season (2).The use of eCG (PMSG) in estrus synchronization protocols in sheep is well established. A single PMSG treatment, after progestin treatment, increases ovarian response, conception rate, and percentage of multiple births from the induced ovulation (14).

There are many advantages to using artificial insemination (AI) in sheep: a ram can mate 50 to 100 ewes per year in a conventional mating program; when we used AI semen collected from a single ram can be used to inseminate over 1000 ewes during 2 to 3 weeks; faster genetic gain for desirable traits in the flock is possible through the use of AI technique (6); AI is the cheapest means to spread

superior genetic traits of elite rams across long distances and beyond the limits of live animals (8); AI eliminates the need for small producers to keep a breeding ram in a herd and also enables them to access semen from superior rams (6), For AI, semen quality is examined on all rams before semen extension and storage, thus sub-fertile males from the flock are remove (8). When using AI there is no direct contact between males and females which helps to minimize disease transmission during breeding; semen can be collected from superior rams which may not be able to breed naturally due to injury, ram-ewe preference during breeding is eliminated in AI (6). AI enables the collection of high quality semen during the breeding season and use it to inseminate synchronized ewes during off-season (6). There are several factors that can modify the effectiveness of artificial insemination and some of them are mentioned below: the breed (3), age of the ewe (1), the season of insemination (1), the use of fresh, cooled, chilled, frozen semen (3), the labor, the year, time of insemination after estrus synchronization, dose of PMSG used, the extender used, dose of inseminated semen, the method (vaginal, cervical, cervico-uterine or laparoscopic) used, detection of ewes on estrus and the number of AI (11, 10, 7).

The purpose of this research was to perform estrus synchronization and artificial insemination in sheep during the breeding season with progesterone (Chronogest, Maravet) and PMSG (Folligon, Maravet).

Materials and methods

The study was carried out during September 2018 - February 2019 in a sheep flock from Covasna county Romania (lat. 44.4267674, long. 26.102538390000063). A number of 125 ewes from Lacune breed with the age between 1.5 - 5 years were synchronized with intravaginal sponges with progesterone (Chronogest, Maravet) and PMSG (Folligon, Maravet) and after that were artificial inseminated by intracervical method.

The sponge (30 mg fluorogestone) was inserted and kept in situ, in the vagina for a period of 12 days. After that, 500-IU PMSG (Folligon, Maravet) was administered intramuscularly at the time of sponge withdrawal on 12^{th} day. Cervical insemination was performed after ewes showed signs of estrus (restlessness, shaking of tail, slightly swollen vulva, moist and reddish cervical external os) at 53h after the removal of sponges. For artificial insemination was used fresh semen collected from 2 rams witch had higher body weight and with a high quality sperm. The semen was collected by the artificial vagina method and after that, a 1/1 dilution of the sperm was performed. After collection, the semen samples were evaluated for volume, consistency, wave motion (0–5 scale), density and percentage of motile spermatozoa (0–100%). After evaluation, a second dilution of the semen samples was performed and finally the samples were 1/10 diluted. The artificial insemination was performed with 0.25 ml of seminal material by intracervical method. At 45 days

after the insemination the pregnancy diagnostic was performed using the transabdominal method with a sectorial probe using the frequency of 5 mH.

Results and discussions

The estrus synchronization and fixed-time artificial insemination were performed in ewes under field conditions of Covasna. The results are presented in table 1.

Table 1

The estrus synchronization and fixed-time artificial insemination in ewes under field conditions of Covasna county (Romania)

Indicator	Number (percentage)
Total animals	125 (100)
Sponge in	125 (100)
Sponge out	123 (98,04)
Ewes in estrus	118 (94,4)
A.I. ewes	118 (94,4)
Lambing of total ewes	102 (81,06)
Lambing of A.I ewes	102 (86,44)
Number of lambs (prolificacy)	132 (105,6)
Specificity of the ultrasound diagnosis	102 (100)

A number of 125 ewes were synchronized with intravaginal sponges. After 12 days a number of 123 (98.04%) sponges were collected, 1.96% sponges had fallen out during this period. All the ewes were intramuscularly injected with hCG. At 53h after the administration of hCG 94.4% cases showed heat sign, so, this animals were artificial inseminated by intracervical method. At 45 days after the artificial insemination the gestation diagnosis was performed for all inseminated ewes. One hundred two diagnosis of gestation were set at 45 days by ultrasound method. The lambing percentage from all the ewes included in the study was 81.06% (102) and the lambing percentage from the ewes artificial inseminated was 86.44%. From 102 gestations a number of 132 lambs was obtained, in 3 cases was observed trigeminal birth and in 23 cases was noted a twin birth. In this situation, the prolificacy was 129.41%.

In this study the percentage of sponge out after synchronization (12 days) was 98.04%, higher than in a study of 2014 (12) where the percentage of sponge

out was 96.07%. In the present study, we found 94.4% ewes showed estrus signs following progesterone sponge removal and eCG injection. Kalyan et al. (12) also reported 79.4% of estrus in ewes following the application of intra-vaginal sponges containing 30–40 mg of fluorogestone acetate (FGA) for 12–14 days and, administration of 500 IU of PMSG in mating season. The results of cervical insemination with fresh diluted semen were acceptable, medium gestation percent being by 86.44%. Kukovics et al. (13) also reported that freshly diluted semen gave the best result 70 to 82%. The accuracy of the gestation diagnosis was 100%, Waterhouse et al. said that the experienced examiner can expect an accurate diagnosis of 91-100% (15). The prolificacy was 105.06% lower that was reported by Fukoi et al. (9).

Conclusions

The results of estrus synchronization and artificial cervical insemination with fresh diluted semen were acceptable, 94.4% of the ewes showed estrus signs, gestation percentage was 86.44% with a prolificacy of 129.41%. The accuracy of the gestation diagnosis was 100%. Estrus synchronization and artificial insemination can be performed with good results in Lacaune ewes.

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PREVALENCE OF CLAW DISORDERS IN DAIRY FARMS WITH TIE STALLS

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Summary

In intensive rearing conditions, dairy cows are exposed to many factors that can cause health disorders and significant economic loses. Today, claw diseases are the main problem in high-milk cow's herd, along with metabolic diseases, mastitis and reproduction disorders. Claw diseases can have direct effects on reproductive parameters. The aim of our research was to determine the frequency of certain diseases of the locomotor apparatus of dairy cows on farms with tie stall system. In the period of two years, a total of 37,893 cows were examined, wherein the following has been found: Laminitis in 34,217 cows (90.30%), Dermatitis interdigitalis in 25,876 cows (68.29%), Dermatitis digitalis in 11,817 cows (31.18%), Rusterholz ulcer in 8,272 cows (21.83%), Fibroma in 3063 cows (8.08%), and Panaritium in 618 cows (1.63%). The results show that laminitis dominate in the herds. Considering the etiology of diseases determined at the farms it is primarily to focus on preventing the formation of metabolic disorders and adequate nutrition of the animals, and then on the improvement of housing conditions and the regular implementation of measures to prevent the spread of infectious claw diseases.

Keywords: dairy cows, claw diseases, tie-stall system

In intensive production cows are exposed to the numerous factors that can cause health problems. Nutrition based on high-energy feed with the addition of large amounts of proteins, poor housing conditions in tie stall system, increased body weight and even the size of the cow milk of high– productive race, they all present a great threat to the health of cows. Based on the large number of studies, about 60% of cows in herds annually have problems related to locomotor tract, followed by a smaller or larger degree of lameness.

Today, the claw diseases are the biggest problem in high productive dairy herds and they affect economic parameters, together with metabolic diseases, mastitis and reproductive disorders. Reproductive problems are still considered as the most important factor for economic losses and, at the same time, it is a quite clear that many reproductive problems arise precisely due to acropodic diseases.

Any stage of the lameness in the first 30 days after delivery causes reduced production and reproductive capacity of the animal. There is a high likelihood that, in such animals, the heat will be concealed and not manifested by clear clinical symptoms.

Claw diseases cause significant direct and indirect damages. Direct damages are due to the cost of the therapy and the inability to use milk due to the withdrawal period after the therapy, as well as due to the forced slaughter of animals that cannot be cured (1, 2, 9). Indirect damage is caused by reduced production of milk in lactation, reduced fitness, reproductive disorders and predisposition for mastitis appearance (5).

Lameness is the earliest and the most important clinical symptom of acropodium diseases of cattle. In 90% of cases the cause of lameness is in claws, and only in 10% of cases in other anatomical parts of the limb. Likewise, in 90% of the cases of lameness, the pathological process is at the hind extremities, in the area of the outer claw, while on the front extremities the process is located on the inner claw.

In tie-system, which is still largely represented in Serbia, the hind extremities are in significantly less hygienic conditions than the front ones, and they are often exposed to mechanical influences (8). In most cases, the primary pathological process is aseptic pododematitis (laminitis). In some farms, it causes more than 80% cases of lameness. When the process take a chronic course, deformities of the claw's corium and the sole occur, followed by secondary infections (8, 6, 5).

The aim of this study was to determine the frequency of certain claw diseases of dairy cows in farms with tie stalls system, with particular review to laminitis.

Materials and methods

At seven dairy farms with tie stalls housing system the condition of claws of lactating cows was monitored. During two years totally 37,893 cows were examined. The checkup and diagnostics were performed during routine processing of claws, twice a year (in total four processing cycles). The obtained data were statistically processed.

Results and discussions

According to the results, at dairy farms in the two-year period Laminitis dominated, which was diagnosed in total 34,217 cows (90.30%), followed by Dermatitis interdigitalis in 25,876 (68.28%), Dermatitis digitalis at 11.817 (31.18%), Rusterholz disease 8,272 (21.83%), Fibroma in 3,063 (8.08%) and Panaritium in 618 cows (1.63%).

Table 1

The prevalence of claw diseases in two consecutive years and two cycles of trimming per year

Farm	Year	Cycle	Diagnosis (%)*						
			L	Rh	Did	Dd	P	F	PS
1	1	1	88.87	13.62	68.82	13.50	2.14	4.81	-
		2	92.74	21.77	61.65	25.87	1.34	7.96	-
	2	1	94.06	14.44	71.10	29.71	1.32	4.79	-
		2	89.62	26.84	74.97	28.01	1.17	8.20	1.81
2	1	1	88.17	11.53	72.34	16.63	1.04	5.62	-
		2	89.79	23.24	69.58	28.16	0.9	9.48	-
	2	1	87.94	24.11	76.27	31.24	1.33	8.14	2.89
		2	97.44	30.06	60.26	45.88	0.82	9.12	3.38
3	1	1	69.18	21.28	49.40	47.73	-	-	-
		2	92.79	14.24	72.42	23.32	0.59	7.46	-
	2	1	90.97	29.15	66.66	32.41	6.43	1.16	-
		2	93.57	27.5	76.24	27.92	1.44	8.66	1.68
4	1	1	88.68	18.93	54.59	15.00	0.54	3.45	-
		2	91.07	10.48	76.60	28.25	0.40	5.32	-
	2	1	90.57	17.45	72.42	35.17	5.27	0.86	-
		2	91.46	18.55	70.36	30.78	0.61	4.83	1.58
5	1	1	79.29	13.38	66.70	33.24	1.95	13.84	-
		2	89.03	30.55	58.62	40.99	1.25	9.45	-
	2	1	96.82	19.98	71.08	33.38	1.43	9.09	-
		2	96.50	33.25	70.70	34.03	1.63	15.77	3.96
6	1	1	78.28	12.36	62.56	36.37	0.95	8.11	-
		2	91.05	31.84	63.68	43.82	1.36	7.88	-
	2	1	89.80	17.67	65.54	37.9	1.94	10.11	-
		2	95.65	33.52	73.23	38.93	2.63	12.95	5.76
7	1	1	90.33	11.90	81.79	28.99	0.71	11.39	-
		2	90.70	25.61	63.71	28.71	4.92	13.30	-
	2	1	100	13.74	75.08	35.9	0.75	10.76	-
		2	92.84	24.9	67.76	25.49	1.06	20.31	2.22

*L - *Laminitis*, Rh - *Rusterholz ulcus*, Did - *Dermatitis interdigitalis*, Dd - *Dermatitis digitalis*, P- *Panaritium*, F - *Fibroma*, PS- *Pododematitis septica*

Laminitis is a huge problem because mostly cows with high production get sick. It is not an infectious disease, such as interdigital dermatitis, digital dermatitis and interdigital phlegmon (foot rot). Laminitis is a metabolic disease with degenerative changes in the corium and claw's wall (the process infiltrates papillae of the corium), and it appears in period around calving. Laminitis does not last for a long time (for several weeks mainly), but walking problems can be prolonged due to deformity of the claws (8).

Causes of laminitis are stress in calving, unbalanced nutrition and overloading of claws. A sudden change in the meal can lead to laminitis, especially

the high content of easily digestible low-fiber carbohydrates, as well as an inadequate rumen development. Rumen acidosis (followed by lactic acid bacteria development and reduction of gram-negative bacteria) accelerates the release of endotoxins. As a consequence, the cow organism produces histamine that first causes vasoconstriction and then vasodilation of the claw's corium capillaries. There are a swelling, hyperemia and destruction of the blood vessels of the corium presented. Claws become painful as the consequence of capillaries' damage which decrease the synthesis of keratin of the wall. Also, ketosis, poor body condition, hormonal changes in the period of calving, udder edema, retention of placenta and dystocia may be the cause of laminitis. Metritis and mastitis may also lead to laminitis development: toxins from the microorganisms in udder via blood can reach the claws and cause the problems in wall development and hemorrhages. Diseases such as interdigital dermatitis, digital dermatitis, IBR (Infectious Bovine Rhinotracheitis) and BVD (Bovine Viral Diarrhea) have been associated with the onset of laminitis (9).

Endotoxins are considered to be a key etiological factor linking nutrition errors, acidosis of the rumen and particular systemic diseases. The source of endotoxins are gram-negative bacteria. When in the lumen of the fore-stomachs a larger amount of easily digestible carbohydrates enters, they start to decompose due to amylolytic bacteria and release the large quantity of organic acids, of which the most important is lactic acid. The acid decreases pH of the rumen's content and this causes an increased disintegration of gram negative bacteria' body and the release of endotoxins. At the same time, permeability of the cutaneous mucous membrane of the rumen is disturbed, which further facilitates the resorption of endotoxins present in the content.

In acidotic conditions, in rumen content the process of decarboxylation of amino-acids is intensified and so histidine generates large amounts of histamine. By reducing the pH of the content, the activity of histaminase in the wall of the rumen is reduced. Therefore, much higher amounts of histamine reach the liver and from there go into systemic circulation. Disorders in histamine-induced circulation confirm the hypothesis of histamine intoxication in rumen acidosis (4, 8).

In etiology and pathogenesis of laminitis, as one of the important predisposing factors, especially at the onset of the disease, the anatomical characteristics of blood vessels in corium which is constricted between the falangeal bone and the wall are mentioned. In such filled space there is no possibility of spreading the vessels in the case of fluid leakage into the interstitium, which is one of the earliest disorders that arises as a result histamine effect on the vascular elements of the claws' corium. Increased transduction and later exudation cause an increase of tissue' pressure that further complicates the blood circulation in laminas and causes their ischemia. This is quite understandable if consider the inelasticity of the corium which cannot be spread under the pressure of created swelling of the claw's corium. At this stage, laminitis is followed by increased temperature and pain of the claw and a high degree of lameness from the onset of the disease. The leaves of the corium, about 1300 in each of the claws, are narrow

and very delicate. They are well-supplied with blood and have a lot of arteriovenous anastomosis. In the tissue of the corium, a rich network of nerve plexuses is spreading, because as it known, the claws are not just mechanical support for the body, but they represent a specific tactile organ (they are involved in maintaining balance and motion). Other actors, such as body weight, body condition, hereditary and acquired anomalies, they can all play a certain role in the pathogenesis of laminitis. Circulation, ischemia and hypoxia disorders cause degenerative changes in the corium and damage of laminas. When the process takes chronic form, a change in the position of the falangeal bone may occur, deformation of the claw and breaking the top of the bone through the sole. These are mechanical damages due to pressure of the cornea lamina, circulation disturbances and the resulting necrotic processes. Related to this, there are a number of changes that occur later, such as thickening of the sole (double sole), bleeding in the sole, and sometimes the appearance of a hematoma. In some cases, this process is characterized by the changes in the color of the sole. Pale fields are often mottled by hyperemic areas or many hemorrhages (1, 2, 3, 4, 8).

On the corium is noticeable the redness, serofibrous to hemorrhagic exudate, bleeding on the surface of the sole surface, especially haemorrhage, cell infiltration and necrosis of the corium, and thrombosis of small blood vessels. In subcutaneous cases, there are histiocytes and fibrosis. In chronic cases, there are deformities of the wall and corium and changes in the position of the bone. A histological finding reveals the process of sclerosing and arising of the perineural connective tissue (4).

In the acute phase of the disease, depending on the degree of inflammation, localization, and intensity of the pain, there is an inability or avoidance of reliance on the claws, which are tempered, diffusely sensitive to pressure, and sometimes with redness and swelling of the corneal margin. In the case of diseased fore claws, the animal kneel on the carpal joints for long period while getting up, so they take food in such a position. When standing and walking in order to relieve the tip of the claw the forelegs are a forward stroke, the walk is reluctant, prolonged and stiff, with short steps. In affected hind claws, the animals are raised to the forelegs and occupy the dog's sitting position or their back is hunched, the hind legs are noticeable under the body and the claws are spared by abduction or leg induction. The heavily diseased animals do not get up, they lie on the side or on the chests with as much possible relief of the hind and the forelegs. This condition is often accompanied by more rapid pulse (up to 120 per minute), fast breathing (up to 80 per minute), increased body temperature (up to 40.5°C) muscle trembling, mucous membrane hemorrhage, decreased or stopped appetite, sweating and, depending on the causes the presence of digestive tract disorders (increased or decreased by number of rumen contraction or diarrhea), puerperal disorders, mastitis, polyarthritis and/or polysynovitis. In sub-acute cases, the symptoms described are less pronounced (1, 2, 3, 4, 8).

Chronic or recurrent cases arise from untreated or untreated acute and sub-acute cases. On the claw’s wall dents are formed (ring changes more or less

parallel with the coronary edge). In addition, the softening of the wall may appeared, with creation of yellow or partially reddish changes on the sole of the claw. The walk is rigid and the load is predominantly on the heel portion of the claw. Animals lose weight. The easier acute cases, after removing the cause, they are cured for one to two weeks. Serious cases, if not treated, often became chronically, with the possibility of complications in the form of taking off the claw, breakthroughs of the sole, infection with the onset of rotten or necrotic pododermatitis. Decubitus wounds and phlegmons, claws' and bone deformities, chronic lameness, progressive weight loss and total exhaustion of the animal appear later (1, 2, 3, 4, 8).

Anamnestic data and clinical picture are sufficient to set the diagnosis. Examination should include the discontinuation of individual claw, "stall claws", coronary margin disease, interdigital phlegmone, claw bone fracture, joint distortion and osteomalatia. In fattening cattle, rickets should be excluded considering the same clinical picture as pododermatitis. The difference is that in rickets the changes are located in long bones (thickening of the epiphysic-diaphragic border), and in pododermatitris they are in claw's corium (8).

Laminitis usually occurs on the hind lateral claw. Acute laminitis appears suddenly and is accompanied by swelling and severe pain. Heart rate and ventilation of the lungs are increased, the claw is heated and swelling over the coronary side of the claw is presented. Hemorrhages are sometimes visible on a white line, or at the passage of the wall into the sole. Sometimes hemorrhage is not visible, causing segregation on the white line, the entry of dirt and the formation of abscess. Subclinical laminitis can last 1 to 3 months. Symptoms of the disease can be seen only after a few weeks or months, based on changes in the wall and in the form of the claw. The most recognizable changes caused by laminitis are: thrived fingers (resulting in increased creation of the horn, with the incorrect growth rings falling backwards; because of this, the outer claw becomes too high and overburdened so that the pain arises, and consequently the unnatural position of the hind legs, as well as limping), the occurrence of hemorrhage in the sole (due to pathological changes in the wall the blood clotting and spraying of the capillaries occur, so the blood is visible during the claw trimming), defect of the white line (the white line can change color from yellow to red, and may be ulcerated), the appearance of a double sole (in severe cases, the sole can be completely separated, often developing a new sole). Chronic laminitis develops without symptoms for 1 to 3 months, then lesions appear on the sole and the wall of the claw, and then a soft claws with unpigmented areas with yellow and haemorrhagic streaks (4, 8).

In acute form, good results are achieved by administering an antihistamine. The most important preventative measure in relation to laminitis is minimizing cows' stress on calving by keeping them in special groups and feeding with balanced meals. It is necessary to use the seeds of proven bulls for easy calving. A good hygienic conditions for cows need to be provided (4, 5).

Conclusions

In a two-year study, laminitis was found to be the most serious health problem on dairy farms with tie-stall system. Infectious diseases of claws are also largely represented (interdigital and digital dermatitis). Considering the etiology of detected claw diseases, it is primarily to focus on preventing the development of metabolic disorders and proper nutrition of the cows, and then on the improvement of housing conditions and the regular implementation of measures to prevent the spread of claw diseases pathogens.

Conflict of interest statement

The authors have no conflict of interest.

Acknowledgements

This research was financed by Ministry of Education, Science and Technological Development of Serbia, Grants TR 31071.

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HISTOLOGICAL CHANGES IN RAT KIDNEYS AFTER *LYCIUM BARBARUM* AND *RUMEX CRISPUS* AQUEOUS EXTRACTS ADMINISTRATION IN ALLOXAN-INDUCED DIABETES

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Summary

Currently, the prevalence of diabetes mellitus, as a metabolic disorder, either type I or type II, is steadily increasing, affecting both human and pets alike. Even if diabetes has as its starting point the pancreas, it will sooner or later affect other organs, injuries known as complications of this disease. The aim of this experiment was to highlight the histological changes in kidneys after seven weeks of *Lycium barbaricum* and *Rumex crispus* aqueous extracts consumption in rats with diabetes induced through intravenous administration of alloxan. The plants taken in study are known to have hypoglycemic effects. The aqueous extract of *Rumex crispus* can not compensate alone the renal structural changes induced by diabetes but the *Lycium barbarum* extract can be used as a protector, the reparatory effects being obvious.

Keywords: diabetes, kidneys, plants, histological changes

Diabetes mellitus is a metabolic disease with multiple etiologies, characterized by chronic hyperglycemia due to an absolute or relative deficiency of insulin (2).

The development of diabetes involves several pathological processes, which include processes that destroyed the beta cells of the pancreas resulting in inability of body tissues and organs to use glucose, which determines the body resistance to insulin. Due to the deficiency of insulin in the body occurs inefficient use of glucose, which induced its accumulation in the circulation, respectively the hyperglycemia. As a result of the accumulation of glucose in the blood plasma, the ability of uriniferous tubules to reabsorb the filtered glucose decreases, resulting in glycosuria. In time, the utilization of glucose in the tissues decreases, compensating for the mobilization and use of the body fat deposit (2, 4).

The main clinical symptoms of diabetes are polydipsia and polyphagia, followed by polyuria. In some cases, although the animals consume more food they tend to lose weight (type I diabetes), or in other cases the obesity will be noted (type II diabetes). Laboratory findings are represented by hyperglycemia, glycosuria and ketonemia (2, 4). In some cases, as subclinical diabetes, there are no clinical symptoms, and the disease is discovered during routine laboratory examination (2). But, the long-term complications (nephropathy, neuropathy, and retinopathy) represent the bigger problem of the diabetes mellitus.

Recently, the use of medicinal herbs in the prevention or even the treatment of metabolic diseases has grown, given the fact that the plants, themselves, are less toxic

and free of side effects in compared to the synthetic ones (1).

Goji berry (*Lycium barbarum*) is considered a "superfood" because of the many bioactive components, with antioxidant and nutritive properties, which include the carbohydrates (46%), fibers (16%), proteins (13%), vitamins, mineral ions and fat (1.5%). The fruits present numerous antioxidant phytochemicals with protective effects in the cases of retinopathy, neuropathy, nephropathy, cardiopathy. Due to the antioxidants components with preapoptotic and antiproliferative activity, Goji berry fruits present an anticancer, immunostimulatory, and modulatory effects. The purified constituents from Goji berry may be involved in lipid metabolism, having a lipid-lowering effect, and in carbohydrate metabolism, having a hypoglycemic effect. Also, some components, like betaine, zeaxanthin present antiaging effects, being anti-inflammatory and sun screen agents (6, 8, 14, 16).

Yellow dock (*Rumex crispus*) is used as a plant with multiple properties, expressed by diuretic, antibacterial, antiviral, anti-diarrheal, anti-anemic and appetite stimulant effects. The plant is also used to relieve pain and swelling, fever, inflammation of the upper respiratory tract, is beneficial in the treatment of sinusitis and used in C hypovitaminosis (10, 12, 15).

The alloxan is an organic compound, which is utilized as diabetogenic agent, being considered a common model for evaluation of the glycemic-control potential of therapeutic drugs and medicinal plants extracts in experimental studies. This urea derivative presents two pathological effects, respectively the selective inhibition of glucose-stimulated insulin secretion, and the induced formation of reactive oxygen species, which determined necrosis of beta cells of the pancreas, and ends with the onset of diabetes type I (5, 7, 9).

Materials and methods

The study design has been extensively described in our previous research (3, 11, 13). Therefore, quite succinctly, the steps of the experiment were the following:

- 25 Wistar albino rats from Animal House of University of Medicine and Pharmacy Victor Babeș Timișoara were kept for acclimatisation during one week, in proper conditions, according with the standard guide for laboratory animals.
- The experimental rats were injected with alloxan 2% (intravenous administration in tail vein) and the installing of hyperglycemia was verified using portable glucometer ACCU-CHEK Active.
- After the onset of diabetes, affected rats were grouped as follows: *C group*, with healthy rats, which received distilled water; *D group*, with induced diabetic rats which also received distilled water until the end of the experiment; *L group*, with induced diabetic rats which received aqueous extract of goji berry (*Lycium barbarum*) in 10% concentration; *R group*, with induced diabetic rats which received aqueous extract of yellow dock (*Rumex crispus*), in 10% concentration; *RL group*, with induced diabetic rats which received aqueous mix made of both plants extracts.

- The experimental rats received aqueous extracts for seven weeks, being carefully monitored during that period.
- At the end of the experiment, the rats were sacrificed, according to current regulations and standards regarding the animal protection.

For histological investigations the pancreas and kidney for each group were collected. The organs were fixated in ethanol 80^0 for seven days, after which they were dehydrated, using ethanol baths, in increased concentrations (80^0, 96^0, absolute) and embedded in paraffin. The histological sections with 5 µm in thickness were obtained using the microtome and were stained by Hematoxyline and Eosin standard method.

The histological images were captured by Olympus CX41 microscope software.

Results and discussions

The histological examination of the pancreas in the control group revealed the normal structural aspect. Thus, the two secretory units, exocrine and endocrine, were clearly observed, since the pancreas is an amphicrine gland, separated in lobes by extremely delicate connective septa, with a dense network of capillaries (Fig. 1). The exocrine component presents pyramidal cells, disposed around a narrow lumen, in the form of serous acini, which continues with a tubular channel system with distribution and collection function. Exocrine cells secrete numerous enzymes that play a role in digestion completion (amylase, trypsin, lipase, collagenase, elastase, etc.). The endocrine component is represented by the Langerhans islands, of ovoid or spherical shape and of different sizes. Endocrine cells, classified as alpha (acidophilic or A), which synthesized glucagon, beta (basophil or B) are the most widely and synthesizing insulin, C (immature cells, precursors of other cell types) and D, which synthesized somatostatin, were arranged in irregular cords or nests.

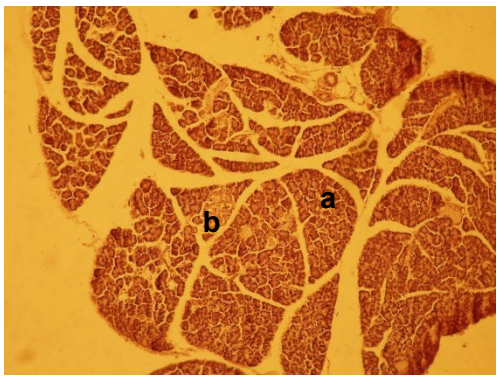

Fig. 1. Histological examination of the pancreas in the control group: normal aspect: exocrine (a) and endocrine components (b), col. Hematoxylin - eosin, 40X

The histological examination of the kidney in the control group revealed a well-structured microscopic architecture, whose parenchyma is divided into two zones: the cortical zone, with renal corpuscles and the medulary zone, with the collecting uriniferous tubules. The nephrons, as structural and functional units of the kidney, contain a fenestrated capillary network, called the vascular glomerus and a canalicular system, represented by the Bowman capsule, the proximal convoluted tubule, loop of Henle and the distal convoluted tubule. The renal corpuscles in which blood plasma filtration takes place are spherical, of different sizes and each present a vascular glomerulus surrounded by a Bowman capsule. The vascular glomerulus detaches from the renal afferent arteriole and continues with the renal efferent arteriole. Among the capillaries are intraglomerular mesangial cells with a role in phagocytosis and/or network contraction. The Bowman capsule present two layers: a parietal one, lined with simple epithelium, supported on a thicker basal membrane, and a visceral layer lined with a simple epithelium with special cells called podocytes. Between the two layers the urinary space is formed (Fig. 2).

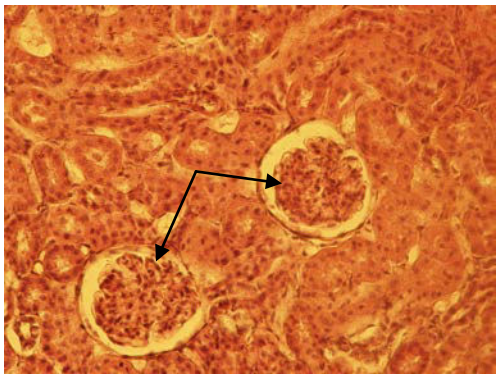

Fig. 2. Histological examination of the kidney in the control group: renal corpuscle with vascular glomerulus (→), col. Hematoxylin - eosin, 100X

The histological examination of the pancreas in the diabetic group induced by the intravenous administration of alloxan showed the occurrence of characteristic degenerative phenomena, ascertained and described earlier in our research, as well as in the literature, among which are: Langerhans islands of different dimensions, the presence of lymphocyte infiltration in some Langerhans islands, known as insulitis, suggesting the involvement of an autoimmune mechanism, and as response following the necrosis of pancreatic endocrine cells (1, 3, 7, 9). Also, in the Langerhans islands where there was necrosis (cytolysis) of the beta-pancreatic endocrine cells, edema areas were installed, which also confers their large size. The more extensive necrotic lesions that affect endocrine cells are, the swelling areas are wider (Fig. 3).

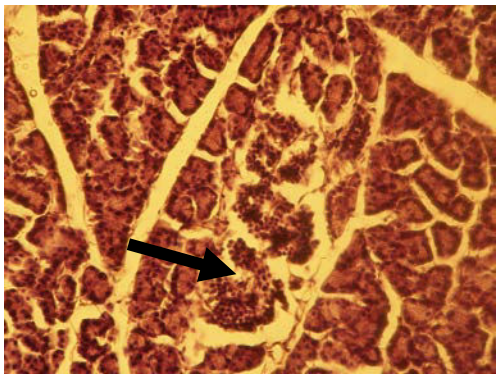

Fig. 3. Histological examination of the pancreas in the diabetic group: lymphocyte infiltration (→), col. Hematoxylin - eosin, 400X

The histological examination of the kidney in the diabetic group induced by the intravenous administration of alloxan revealed the occurrence of vascular congestive phenomena, more evident in the medulary zone and degenerative phenomena at the level of renal corpuscles and uriniferous tubules (Fig. 4).

Vascular hypertrophy or congestion, according to the literature, is a progressive complication that will lead to hypertension and ischemic nephropathy.

Also, there was a reduction in the urinary space of the renal corpuscles, probably due to a higher blood flow, which results in dilatation of the glomeruli and / or due to the presence of leukocyte infiltrate both at their level and peritubular (Fig. 4).

Extended leukocyte infiltrate indicates interstitial nephritis.

Also, at the level of the proximal convoluted tubules, the nephrocytes showed a turgescent appearance with microvilli destruction, in some areas nephrosis and even tubular necrosis. The turgescence of the nephrocytes may indicate a hydropic degeneration, a lesion considered to be reversible.

These degenerative phenomena affect the function of the proximal convoluted tubules involved in the reabsorption of 90% of the blood filtrate.

The microscopic lesions identified at the kidney level are similar to those found in the literature.

The histological examination of the kidney of the group with alloxan-induced diabetes and treated with the Goji Berry aqueous extract revealed the existence of vascular phenomena with congestion and thickening of the arterial walls.

At the level of renal corpuscles, although the urinary space was still reduced, there was no leukocyte infiltrate, indicating the recovery of the blood plasma filtration function, and the reduction of the tubular nephrosis phenomenon, especially from the proximal convoluted tubules, which means the recovery of the absorption (Fig. 5). Therefore, Goji Berry aqueous extract can be used as a renal protector.

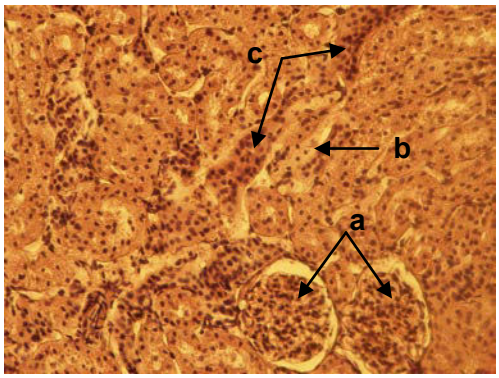

Fig. 4. Histological examination of the kidney in the diabetic group: leukocytes infiltration in the vascular glomeruli (a) and peritubular (b), tubular nephrosis (c), col. Hematoxylin - eosin, 400X

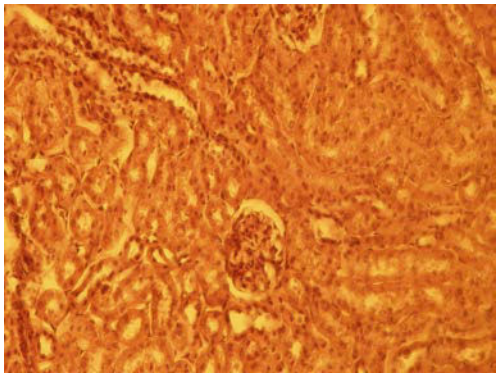

Fig. 5. Histological examination of the kidney in the group with diabetes induced by intravenous administration of alloxan, and treated with aqueous Goji Berry extract: absence of the leukocytes infiltration on the vascular glomeruli and peritubular level, col. Hematoxylin - eosin, 400X

Our previous research on the use of Goji Berry extract has highlighted that although these fruits are able to reduce blood glucose, the only repair / compensatory phenomenon was vascular hyperemia, which provides nutritional support to exo and endocrine glandular cells of the pancreas.

The histological examination of the kidney in the diabetic group induced by intravenous administration of alloxan and treated with the extract of *Rumex crispus* revealed an increase in the infiltrative phenomena of the vascular glomeruli accompanied by nephrocyte necrosis in the proximal convoluted tubules (Fig. 6).

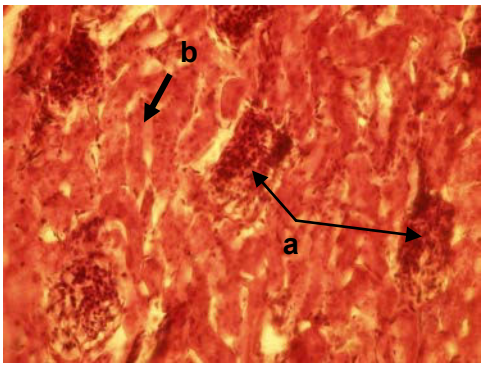

Fig. 6. Histological examination of the kidney in the group with diabetes induced by intravenous administration of alloxan, and treated with Yellow dock aqueous extract: leukocyte infiltrate in the vascular glomeruli (a), acute tubular necrosis (b) col. Hematoxylin - eosin, 400X

Therefore, this aqueous extract cannot alone compensate the effects of alloxan administration.

Conclusions

Intravenous administration of alloxan determines the installation of injuries severe enough to be difficult to compensate, at the pancreas and kidney levels. The aqueous extracts of Goji Berry (*Lycium barbarum*) used in the treatment of kidney injuries induced by intravenous administration of alloxan present a renal protective effect, the reparatory effects being obvious.

The aqueous extract of Yellow dock (*Rumex Crispus*) alone cannot compensate for the effects induced by diabetes installation in the kidney.

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A PROCEDURE FOR IDENTIFYING GENETICALLY MODIFIED ORGANISMS FROM FEED IN COW'S MILK

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Summary

In cow's milk there are fragments of the animal DNA (which passes into the secretion product together with the somatic cells), as well as fragments of plant DNA (taken from the fodder into the blood and then into the mammary gland from where it passes into milk). This paper presents an experiment attempting to develop an exogenous DNA that is found in milk samples analysis procedure, with the goal of identifying genetically modified organisms that can be introduced into farm animal feed. Milk samples were collected from the commerce but also from cows farms. The experiment began by filtering milk samples and continued with DNA purification. The DNA obtained was analyzed by PCR based methods, which involved the amplification of the genes of interest. PCR products were analyzed by migration to agarose gel, the presence of a specific product allowing qualitative assessment of the results.

Keywords: milk, exogenous DNA, PCR analysis, genetically modified organisms

How can exogenous DNA pass into milk? Ingested fodder is mechanically disrupted; the DNA is released and cleaved through acid hydrolysis and enzymatic digestion into small DNA sequences and free nucleotides. The activity of various phosphatases and deaminases is further affecting the structural integrity of free DNA. However, small sequences of DNA are absorbed in the circulatory system at the ileum level. DNA fragments arrive with blood in the udder, where they are filtered and subsequently excreted with the secretion product of the mammary gland. (1,4,6)

In this very pathway the transgenic DNA can pass as small fragments in the milk of the animal that are ingesting genetically modified organisms containing feed. All the transgenes are controlled by promoters and terminal regions. They are resistant to insects Lepidoptera and herbicide tolerance based on glyphosate (plants killed by interfering with essential amino acids: phenylalanine, tyrosine and tryptophan). The genetic improvement of the *Roundup Ready Soybean* strain was initially made with: the EPSPS enzyme of the strain CP4 of the *Agrobacterium tumefaciens* bacterium, the *cauliflower mosaic* 35S (E35S) promoter, the chloroplast transit peptide (CTP4) coding sequence from *Petunia hibrida*. Genetically modified maize lines are MON810 and Bt176. *MON810* is enhanced with sequences from: *Bacillus thuringiensis*, *Ochrobactrum anthropi, Agrobacterium tumefaciens* strain CP4, *Escherichia coli*. *Bt176* is enhanced with DNA sequences: *Bacillus thuringiensis kurstaki, Streptomyces hygroscopicus,*

Escherichia coli. Worldwide, 30% of maize crops are genetically modified maize lines, the main cultivators are the USA, Brazil and Argentina. Of the European countries, most have chosen not to cultivate genetically modified organisms, they do it for research purposes only. The genetically modified lines that we want to identify are: RR soybeans by highlighting the "35S" promoter and the "nos" terminal sequence, and the corn lines produced, MON 810 and BT-176, are identified by the "35S" promoter (3, 5, 7).

Materials and methods

The samples used in this study are represented by two samples of pasteurized milk taken from local market, namely samples 1 and 2, and four raw milk all taken from the different local cow farms, namely 3, 4, 5 and 6.

DNA purification. For the beginning the milk samples were diluted 1:4 and after were subjected to microfiltering. Special filters whose pores do not exceed 0.45 microns have retained endogenous DNA (incorporated into somatic cells but also nucleic acids bound to other structures larger than these pores) and allowed to pass free DNA fragments. For the purification of free circulating DNA a commercial kit designed for genomic DNA purification was used (NucleoSpin gDNA Clean-up, Makerey Nagel, Germany).

NucleoSpin gDNA Clean-up work protocol:

1. Adjust DNA binding conditions - 150µl sample + 450µl Binding Buffer ,Vortex five seconds;
2. Bind DNA - Load sample on NucleoSpin gDNA Clean-up Column, 11000*g/30 seconds;
3. Wash silica membrane: 700µl Wash Buffer- Vortex 2s, 11000*g/30s, and 700µl Wash Buffer -Vortex two seconds, 11000*g/30 seconds;
4. Dry silica membrane – 11000*g/ one minute;
5. Elute DNA- 20 µl Elution Buffer, 11000*g/30 seconds.

Qualitative and quantitative assessment of nucleic acids was done by examining a microliter of the DNA solution using the NanoDrop 8000 Spectrophotometer (Thermo Scientific, USA).

The next step is the PCR reaction (2) in which we used the five primers (Table1).

The conditions for PCR amplification were the following:

For RuBisCo gene: denaturing step for three minutes at 95°C, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 63°C for 45 seconds and extension at 72°C for one minute, with a final step at 72°C for three minutes.

For Lectin gene: denaturation 95°C- three minutes; 40 cycles: denaturation 95°C -25 seconds; primer annealing 62°C - 30 seconds, DNA synthesis 72°C - 45 seconds; final extension 72°C – seven minutes;

For T-nos terminator region: denaturation 95°C-three minutes; 40 cycles: denaturation 95°C -30 seconds; primer annealing 63°C - 30 seconds, DNA synthesis 72°C - 30 seconds; final extension 72°C –three minutes.

Table1

PCR primers pairs used in this study

RuBisCo	^{5}CGTAGCTTCCGGTGGTATCCACGT$^{3'}$, $^{5'}$GGGGCAGGTAAGAAAGGGTTTCGTA$^{3'}$
Soybean specific (lectin)	$^{5'}$GCCCTCTACTCCACCCCCATCC$^{3'}$ $^{5'}$GCCCATCTGCAA GCCTTTTTGTG$^{3'}$
GM soybean (nos terminator)	$^{5'}$GCATGACGTTATTTATGAGATGGG^{3} $^{5'}$GACACCGCGCGCGATAATTTATCC$^{3'}$.
GM maize (Promotor 35S CaMV)	*5'*-CCACGTCTTCAAAGCAAGTGG- 3' *5'*- TCCTCTCCAAATGAAATGAACTTCC - 3'
Zea mays specific (zein)	*5'*- AGTGCGACCCATATTCCAG- 3' *5'*- GACATTGTGGCATCATCATT - 3'

For Zein gene: denaturation 95°C-10 minutes; 50 cycles: denaturation 94°C -25 seconds, primer annealing 62°C - 30 seconds, DNA synthesis 72°C - 45 seconds; final extension 72°C – seven minutes.

For Promotor 35 CaMV region: denaturation 95°C-10 minutes; 50 cycles: denaturation 94°C -25 seconds, primer annealing 62°C -30 seconds, DNA synthesis 72° - 45 seconds; final extension 72°C -seven minutes.

The resulting PCR products were separated on 2% agarose gels in TAE buffer at room temperature at a constant voltage of 100 V for 40 minutes. The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England).

Results and discussions

In the first step of the study, total genomic DNA was isolated and purified from the microfiltrated samples. Thus, the spectrophotomeric concentration of the nucleic acids in commercial pasteurized milk did not exceed 4 ng / µl. Since the concentration of nucleic acids in raw milk on the farm varied between 23.76 and 49.18 ng / µl, these samples were brought to the concentration of 20 ng / µl.

DNA of amplifiable quality and quantity was obtained and serial dilutions were prepared in the attempt to equalize the genes of interest copies number that may be present in the DNA samples. As expected, the quantity and quality of isolated DNA from processed samples was lower but still considered suitable for PCR analysis.

RuBisCo is an enzyme involved in plant photosynthesis. By determining its molecular marker, we can certify the presence of vegetal DNA in the sample and

that the present DNA is of amplifiable quality (Fig.1) as it was previously demonstrated by Boldura et all, 2015. Electrophoretic migration in the agarose gel of a DNA fragment of 264 base pairs allows indicated that the purified free circulating DNA it's also becoming from a vegetal source (in this case from the animal's alimentation) but, moreover that this DNA could be amplified in order to detect the composition of cow's alimentary intake.

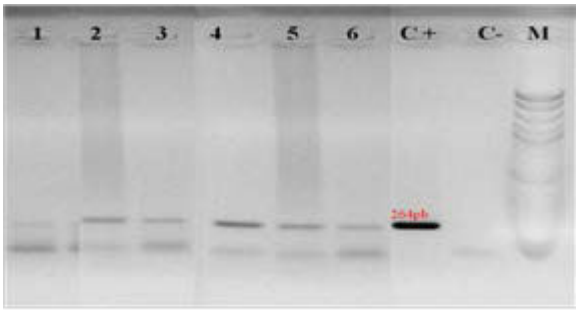

Fig. 1. PCR detection of soybean DNA by detecting RuBisCo specific sequence. Pasteurized milk: 1, 2. Raw milk: 3, 4, 5, 6. Pozitive control: C+. Negative control: C- . DNA ladder, PCR marker (Thermo Scientific™ GeneRuler™50 bp DNA Ladder):M

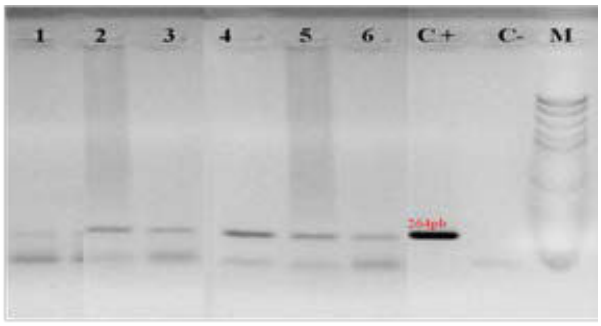

Fig. 1. PCR detection of soybean DNA by detecting RuBisCo specific sequence. Pasteurized milk: 1, 2. Raw milk: 3, 4, 5, 6. Pozitive control: C+. Negative control: C- . DNA ladder, PCR marker (Thermo Scientific™ GeneRuler™50 bp DNA Ladder):M

The next step was to identify whatever the forage of the analyzed individuals might be containing corn. For that the presence of a specific corn gene was detected. Zein is a protein specific to corn (*Zea mays L.*). With the electrophoretic migration of its molecular marker a band of 277 base pairs is outlined. Thus only samples 3, 4, 5, 6 confirm that the cow's forage contains corn

(Fig. 2), enabling the next PCR analysis the detection of genetically modified corn. The results obtained in this case indicate that the corn that is present in the cow's alimentation is not genetically modified, since the amplification for "35 S" promoter, a molecular marker for the genetically modified corn (123 base pairs) is absent for all the samples (Fig. 3) and positive in the case of positive control.

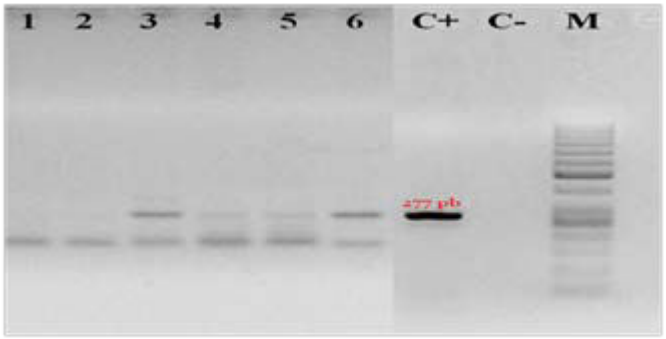

Fig. 2. PCR detection of soybean DNA by detecting Zein specific sequence. Pasteurized milk: 1, 2. Raw milk: 3, 4, 5, 6. Positive control: C+. Negative control: C-, DNA ladder, PCR marker (Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder: M

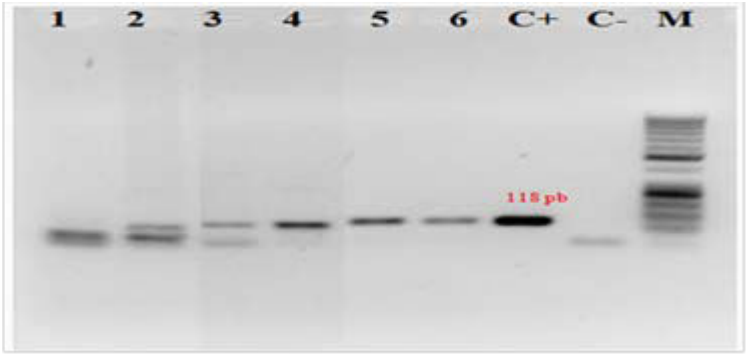

Fig. 3. PCR detection of maize DNA by detecting "35S" promoter specific sequence. Pasteurized milk: 1, 2. Raw milk: 3, 4, 5, 6. Positive control: C+. Negative control: C-. DNA ladder, PCR marker (Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder): M

The same pattern was followed in the case of genetically modified soybean detection. Lectin is the protein specific for this organism and helps in identifying the presence of soy DNA. Its molecular marker has a dimension of 118 base pairs. As

it can be noticed from the Fig. 4, the lectin gene was identified in all analyzed samples.

The genetically modified soy is identified by highlighting the "T nos" terminal sequence which is 118 base pairs long. In this study we were able to identify the presence of genetically modified soybean in two samples, namely sample (pasteurized milk) and sample 3 (raw milk) (Fig. 5).

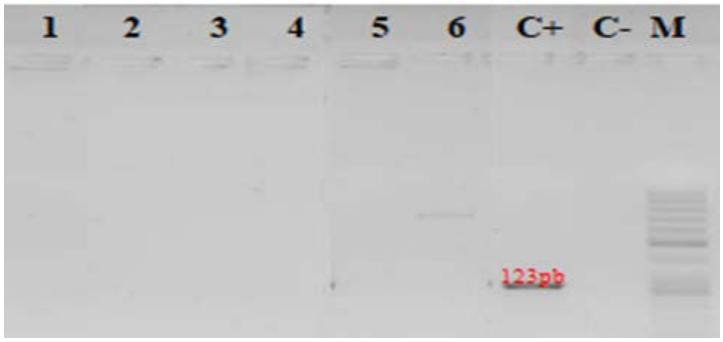

Fig. 4. PCR detection of soybean DNA by detecting lectin specific sequence. Pasteurized milk: 1, 2. Raw milk: 3, 4, 5, 6. Positive control: C+. Negative control: C-. DNA ladder, PCR marker (Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder): M

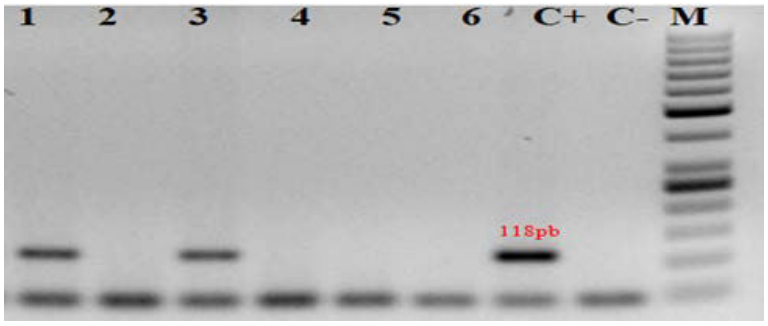

Fig.5. PCR detection of genetically modified soybean DNA by detecting ”Tnos” terminator specific sequence. Pasteurized milk: 1, 2. Raw milk: 3, 4, 5, 6. Positive control: C+. Negative control: C-. DNA ladder, PCR marker (Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder): M

As performing this study we were able to demonstrate that the free circulating DNA can be present in the milk of animals and that probably a large part of this DNA can become from the feed ingested by the animals. Our findings were

similar to those of Phipps et al, 2003 (6), even if the method of DNA isolation and purification was different to the one that was proposed in the bibliographic reference. Considering this, we were able to identify in this solution two species that were part of the feed composition and also, in the case of genetically modified organisms it was possible to detect the transgene. However those are preliminary results, and as it is predicted in the literature, it might be that this detection to be hazardous.

Conclusions

The procedure of microfiltrating the diluted milk samples in order to select only the exogenous DNA, it was successfully used, still we consider that it needs further improvement, since there are no recordings of this protocol in the bibliographic references.

The protocol used allows identification of exogenous DNA in cow's milk by PCR.

With the help of DNA known fragments such as promoters and transgenic termination regions (molecular markers for the transgenes), we can identify genetically modified organisms that have been introduced into the feed of dairy cows.

Acknowledgement

This work was conducted in Laboratories A1c and A2 from Horia Cernescu Research Unit of Banat's University of Agricultural Sciences and Veterinary Medicine „ King Michael I of Romania", Timisoara, Romania.

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IMMUNOLOGICAL ASPECTS ON THE ACTIVITY OF IMMUNOBIOLOGICAL INDICATORS AT BOVINES SOME PHYSIOLOGICAL GROUPS

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Summary

The scientifica investigations reflected in this study presents the dynamic study of some immunobiological blood-type indices characteristic to immunobiological status at bovines some physiological groups during the gestation and postpartum periods. There are presented aspects of immunological indicators, regarding the importance of installing cellular and humoral immunity. Important data presents the values of leukocytes, erythrocytes, lymphocytes and T and B indices of immunocompetent cells at different stages of research. The initiation of these researches revealed some increases of their level, both at pregnant and postpartum bovines, in particular the concentration of T and B lymphocytes, which justifies the importance of cellular and humoral resistance specific to the animal organism in different physiological states.

Keywords: Lymphocytes, pregnant bovines, post partum bovines, T cells, B cells

The key to effectiveness of the immune system of animals and humans is represented by the way in which its constituent network communicates. Millions of well-organized cells continuously transmit information and, in case of an alarm connected to an intruder, these produce chemical substances to fight effectively against the "enemy." A fundamental feature of the immune system is its ability to distinguish between cells of its own organism and foreign cells (3, 4, 10).

In the modern medicine exists methods to calm down the immune system reaction, but were not invented sufficient good methods to be sure of these achievements. Thus, the immune system can be beneficial or harmful for the organism, depending on the nature and power of its reactions. It doesn't exist a fundamental difference between the mechanisms which stay at the base of the "protector immunity" and those which causes diverse immune deceases at animals and man (1, 8).

It is remarkable to mention, that according to some authors, the component elements of the immune system which represent an elaborated, interactive system of cells, chemical substances and tissues distributed in the whole organism. Among the most important component elements of the immune system are lymphocytes (white cells from blood), chemical substances such as cytokines, antibodies and the complement system and tissues, such as lymph nodes ("ganglia"). These form the "cutting margin" of the immune system and represent

instruments which body uses to decompose, destroy and eliminate the harmful antigens such as bacteria, viruses (2, 5).

Thus, the T cells (approximatelly 70% from the lymphocytes) migrates to the thymus where it grows and matures. The T cells contribute to the defense of organism in two ways: regulates the immune system mechanisms and destroys the infected cells.

In the same time, the T cells (aprox.10% from lymphocytes), matures in the bone marrow and other components of the immune system, less the thymus; these cells produce the antibodies (6, 7, 9).

From this point of view, the objective of this research constituted the research of some immunological aspects on the activity of some immunobiological indices at physiological groups of bovines during the gestation and postpartum period.

Materials and methods

The scientific researches were performed in the Microbiology Laboratory of the Faculty of Veterinary Medicine of the State Agrarian University and the Private Laboratory Synevo, from Chisinau. For performing the scientific investigations were used samples of blood from pregnant and postpartum bovines during diverse periods.

The blood samples were collected from the jugular vein with heparin based on the calculation 0.3 ml heparin at 10 ml of blood, with the purpose of anticoagulation. The samples were used to identify the number of leukocytes, erythrocytes, lymphocytes and the indices of T and B lymphocytes immunocompetent cells.

Results and discussion

The obtained results regarding the immunological investigations on the immune system regarding studying some immunological aspects about the activity of some immunobiological indices at physiological groups of bovines in period of gestation and postpartum reveal important immunological indices.

Thus, the results from the table 1, regarding the dynamics of the immunological indices at gestating cattle in different periods of gestation, reveal that at the level of leukocytes, erythrocytes, lymphocytes and indices of immunocompetent T and B cells vary at different stages of research.

Significant results of the leukocytes and erythrocytes indices were registered at gestating bovines up to 10 and 20 days, constituting values of leukocytes equal to 8.67 ± 1.54; 7.12 ± 0.06 and 4.62 ± 0.15; 3.84 ± 0.12, compared to the values obtained at the gestating bovines up to 30 days where the indices constituted the level of 7.84 ± 0.04 and 4.53 ± 0.14.

Simultaneously were determined and appreciated the number of lymphocytes at gestating animals, which reveals appreciable values in period of 10 and 20 days postpartum, constituted 54.83 ± 4.4 and 57.45 ± 6.7, compared to gestating bovines up to 30 days postpartum constituted the lymphocytes indices equal to 56.24 ± 4.2.

Aspects of immunocompetent cells notice important values at bovines from all periods of gestation, determining remarkable indices of T lymphocytes up to 10 and 20 days postpartum which constituted 33.83 ± 6.6 and 38.11 ± 3.7 compared to gestating bovines up to 30 days postpartum which constituted 37.14± 3.4. In the same time important values were determined by the values of the B lymphocytes, which at gestating bovines up to 10 and 20 days postpartum constituted values equal to 10.77 ± 5.2 and 11.89 ± 4.3, compared to gestating bovines up to 30 days postpartum which constituted 12.15 ± 3.4.

Table 1

The dynamics of the immunological indices at gestating bovines in different gestating periods

Age (days)	Number of animals	Leukocytes (10/l)	Erytrocytes (10 /l)	Lymphocytes (%)	T-lymphocytes (%)	B-lymphocytes (%)
Gestating bovines up to:						
-10 days postpartum	5	8.67 ± 1.54	4.62 ± 0.15	54.83 ± 4.4	33.83 ± 6.6	10.77 ±5.2
-20 days postpartum	5	7.12 ± 0.06	3.84 ± 0.12	57.45 ± 6.7	38.11 ± 3.7	11.89± 4.3
-30 days postpartum	5	7.84 ± 0.04	4.53 ± 0.14	56.24 ± 4.2	37.14 ± 3.4	12.15 ± 3.4

The data from the table 2, regarding the dynamics of the immunological indices at postpartum bovines in different periods, reveal that the level of immunological indices at these physiological groups of animals vary depending at which steps of research we are.

Therefore, aspects of these indicia reveal that at the postpartum bovines after 10 and 20 days, were registered values of leukocytes and erythrocytes namely: 7.21 ± 0.03; 8.15 ± 0.05 and 4.31± 0.21; 4.64 ± 0.18, compared to values obtained postpartum after 30 days where the indices constituted 8.32 ± 0.03 and 5.24 ± 0.12.

Simultaneously were determined and appreciated the number of lymphocytes at postpartum animals, which reveals appreciable values in period of 10 and 20 days after postpartum, constituting 53.26 ± 5.8 and 46.13 ± 3.2, compared to bovines after 30 days postpartum constituting the lymphocytic indices equal to 55.87 ± 5.1.

Table 2

The dynamics of the immunological indices at postpartum bovines in different periods

Age (days)	Number of animals	Leukocytes (10/l)	Erytrocytes (10 /l)	Lymphocytes (%)	T-lymphocytes (%)	B-lymphocytes (%)
Postpartum bovines after:						
-10 days postpartum	5	7.21 ± 0.03	4.31 ± 0.21	53.26 ± 5.8	39.84 ± 1.6	12.51 ±2.2
-20 days postpartum	5	8.15 ± 0.05	4.64 ± 0.18	46.13 ± 3.2	42.02 ± 0.7	14.36 ± 3.5
-30 days postpartum	5	8.32 ± 0.03	5.24± 0.12	55.87 ± 5.1	39.61 ± 1.2	15.16 ± 5.5

The aspects of the immunocompetent cells remark important values at bovines from all postpartum periods, determining remarkable indices of the T lymphocytes after 10 and 20 days postpartum which constituted 39.84 ± 1.6 and 42.02 ± 0.7 compared to bovines after 30 days postpartum which constituted 39.61± 1.2. In the same time, important values were determined at the level of B lymphocytes, which at the postpartum bovines after 10 and 20 days constituted values of 12.51 ± 2.2 and 14.36 ± 3.5 compared to postpartum bovines after 30 days where the level of B lymphocytes constitutes 15.16 ± 5.5.

These registered indices reveal some increases at their level both for gestating bovines as well as for postpartum, especially the concentration of the T and B lymphocytes, which justifies the importance of the cell and humoral resistance specific to the immune status of the animal as a result of the installation of the organism immunity. Therefore, as a result of activation B cells are differentiated in plasmocytes and are characterized by the presence of the membrane receptors belonging to the isotypes IgM and IgG.

According to the performed researches, T lymphocytes are responsible for the cell immunity and express the receptors which recognize some parts of the peptides from protein antigens. From this point of view, we may conclude under the fact that the cell base of the cell immune response is represented by the T and B lymphocytes, and thus, the immune cell response protects the animal organism against of fungi, parasites, viruses and bacteria with intracellular localization.

We consider fully argued the idea, that the principal factors of the immunity of animals are represented by the lymphocytes and immunocompetent cells: T and B, which determine the immune reactions of organism and the development of the immunity and of tolerance and is subject to some mechanisms of regulation because the immune response towards antigen or the tolerance towards a potential pathogen can have no favorable consequences for life.

The regulation of humoral or cellular immune response is a complex modulation process in which there are a number of means by which specific organism defense is maintained at a certain level and with a certain duration in order to achieve homeostasis and preserve health.

Based on these considerations, we reveal that these findings allow us to conclude that is taking place the installation of the immunological reactivity of the gestational and postpartum organisms and adaptation to changes in environmental conditions, especially at the action of the pathogens factors.

Conclusions

The evaluation of the immunological aspects on the activity of the immunobiological indices at bovines of some physiological groups offers the possibility to analyze the dynamics of the immunological indices at the gestational and postpartum bovines during different periods, being considered the main ones in the regulation of the immune system.

The results regarding the number of lymphocytes at gestating animals denote appreciable values during the period of 10-20 days postpartum, constituting 54.83 ± 4.4 and 57.45 ± 6.7, compared to gestating bovines up to 30 postpartum days, constituting lymphocytic indices equal to 56.24 ± 4.2.

Values of immunocompetent cells determines important data at bovines of all postpartum periods, resulting in remarkable T cells lymphocytes indices after 10 and 20 days equal to 39.84 ± 1.6 and 42.02 ± 0.7 compared to bovines after 30 days being equal to 39.61 ± 1.2.

The level of B lymphocytes at postpartum bovines determined values equal to 12.51 ± 2.2 and 14.36 ± 3.5 versus postpartum bovines after 30 days, constituting 15.16 ± 5.5.

The dynamics of registered indices reveal some increase in their level at both gestating and postpartum bovines, especially the T and B lymphocytes concentration, which justifies the importance of cellular and humoral resistance specific to the animal's immune status as a result of immune system installation in the organism.

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DNA QUALITY AND QUANTITY DETERMINATION- IMPORTANCE FOR BIOCHEMICAL PRACTICAL APLICATION

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Summary

Molecular methods of laboratory analysis are increasingly based on the use of nucleic acids. These methods find their practical applicability in an increasingly extensive area of laboratory analysis. The success of each analysis starts from the isolation and purification of nucleic acids. A non-compliant sample in this case may lead to the total failure of the biochemical analysis that are to be carried out subsequently. Therefore, a number of methods have been developed and applied, in which the quality and quantity of DNA extracted from different matrices can be evaluated. The present paper presents two of these methods that can be used individually but gives the best information when used together: agarose gel electrophoresis and spectrophotometric analysis of total genomic DNA molecules. DNA was extracted from different matrices using the same method and then analyzed by the two methods. The obtained data were separately interpreted and after a parallel between those methods is presented.

Keywords: DNA quality and quantity, spectrophotometry, gel electrophoresis, molecular data assessment

DNA can be isolated from various sources, such as: calf tissue, blood, animal matrix, plant matrix, bones. The quality and quantity of DNA and RNA are key factors in the success of any analysis to be made (1, 2).

Reliable measurement of DNA concentration and purity is important for many applications in biochemistry and molecular biology, and accurate determination of DNA concentration is considered critical. Impurities present in the DNA suspension can lead to inaccurate measurement of DNA concentration and may inhibit subsequent enzymatic reactions (1, 8).

DNA concentration can be evaluated using four different methods: absorption (optical density), agarose gel electrophoresis, fluorescent DNA binding dyes and luciferase-pyrophosphorylation coupling. However, the most commonly used methods for measuring the purity and concentration of DNA are absorption (as measured by a spectrophotometer) and agarose gel analysis (8).

Spectrophotometry is a method used to measure how much a chemical absorbs light by measuring the intensity of light as a light beam passes through the sample solution. A spectrophotometer is a tool that measures the amount of photons (intensity of light) absorbed after passing through the sample solution. The DNA absorption properties can be used to detect, quantify and evaluate purity (3, 4, 5).

Nucleic acids absorb ultraviolet light (UV) due to the heterocyclic rings of nucleotides, and compounds such as carbohydrates and phosphates do not contribute to this absorption. The wavelength of the maximum absorption for nucleic acids is 260nm (λmax = 260nm) with a characteristic value for each of the individual nitrogen bases. The DNA absorption properties can be used to detect, quantify and evaluate purity.

Electrophoresis is a physicochemical technique whereby charging particles migrate and separate on a particular physical support under the influence of an electric field.

Agarose gel electrophoresis of the purified DNA can complete and straighten the data from the absorption readings (6). Concentration can be determined by completing the gel by comparing the intensity of the DNA sample with that of a DNA quantification standard. The standards used for quantification must be labeled in the same way and be the same size as the analyzed DNA sample. To visualize DNA in the agarose gel, staining with an intercalating dye, such as ethidium bromide, is required. This method is useful for cases where the concentration is too low to accurately assess spectrophotometry and in cases where contaminants that absorb at 260nm make it impossible to accurately quantify by this method (9).

However, the most common reason for running a gel is access to DNA quality. On an agarose gel of 1 to 1.5%, intact genomic DNA should appear as a compact, high molecular weight band with no traces of low molecular weight smears. The latter indicate the presence of the DNA molecule degradation (6).

Materials and methods

The biological material was represented by DNA suspensions, prepared from different matrixes (Table 1). The DNA was previously extracted and purified (data not described here) using the same CTAB based procedure.

Table 1

The biological samples used in the present study

No. crt.	Sample content
1.	Vegetal feed A
2.	Vegetal feed B
3.	Cow muscle tissue
4.	Fish muscle tissue A
5.	Fish muscle tissue B
6.	Horse muscle tissue
7.	Pet food
8.	Calf muscle tissue
9.	Pig liver tissue
10.	Corn flour

The methods used in assessing DNA quantity and purity were agarose gel electrophoresis and spectrophotometric quantitation.

Agarose gel electrophoresis (6, 8)

Necessary materials:

1. Agarose
2. TEA buffer: 0.04 M Tris-acetate; 0.002 M EDTA
3. Ethidium bromide (10 mg / ml)
4. Blue Phenol-xylene Cyanol Migration Solution 10x (for 100 ml solution: 0.25 g blue bromophenol, 0.25 g xylene cyanol and 30 g glycerin are dissolved in 100 ml distilled water).

Procedure:

A horizontal electrophoretic plate is used, as a closed mold. In this mold a comb is inserted for creating the holes in which the DNA samples will be inserted.

1. For gel preparation weigh the required amount of agarose (0.7g / 100ml TEA buffer for isolated and purified genomic DNA). The mixture is heated until the agarose is completely dissolved.
2. Cool the mixture to about 50°C, add 3.5 µl ethidium bromide and mix gently. The solution thus obtained is poured into the mold.
3. Allow the agarose solution to cool in the mold until complete solidification.
4. After cooling the gel, remove the combs, taking care not to deform the wells.
5. The gel thus obtained is introduced into the electrophoresis tank where TEA buffer solution is added until the gel is covered.
6. Add 3 µl of migration dye solution to each 5 µl ADN sample.
7. DNA migration into the gel takes place using a voltage around 90 V for about 40 minutes.
8. The gel DNA was visualized using a UV trans illuminator.
9. With the help of specific equipment, the gel was photographed. For the processing of experimental data, the VisionWorks®LS software (*UVP, England*) was used.

Spectrophotometric quantitation

The amount and quality of isolated DNA were verified by the spectrophotometric method using the Nanodrop 8000 UV-VIS spectrophotometer (Thermo Scientific).

The absorbance at 260nm is used to calculate the concentration of nucleic acids. At a concentration of 50 µg/ml and a 1 cm path length* dsDNA has A260 = 1.

Concentration (µg/ml) = (A260 reading – A320 reading) × dilution factor × 50µg/ml

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume (7).

DNA Yield (µg) = DNA Concentration × Total Sample Volume (ml) (6, 8).

The raw data are interpreted by the equipment software and no further analysis is required.

Results and discussions

For each of the samples analyzed in this experiment data were obtained by both methods of analysis used.

These methods together were summed up so that the data obtained is as complete and accurate as possible.

Spectrophotometric analyzes yielded data that provided information on the amount of nucleic acids present in biological samples, as well as on their purity in terms of contamination with organic solvents and the presence of some protein substances, which both can affect the accuracy of the results (Table 2).

Table 2

Spectrophotometric results on DNA concentration and purity

No. crt.	Sample description	DNA concentration (ng/µl)	Abs Ratio 260/280	Abs Ratio 260/230
1.	Vegetal feed A	533.2	2.01	2.2
2.	Vegetal feed B	289.4	2.01	2.35
3.	Cow muscle tissue	34.03	1.89	3.04
4.	Fish muscle tissue A	36.1	1.91	1.85
5.	Fish muscle tissue B	27.5	1.77	0.83
6.	Horse muscle tissue	23.2	1.59	1.03
7.	Pet food	310.3	2.07	2.31
8.	Calf muscle tissue	551.9	1.68	2.10
9.	Pig liver tissue	676.3	1.84	2.35
10.	Corn flour	25.3	1.78	1.85

After the spectrophotometric analysis of the DNA suspensions, they were analyzed by agarose gel electrophoresis. Although this method allows for the quantification of nucleic acids by comparing them with a molecular weight marker, in this study we used this method to assess the degree of DNA molecule degradation.

As for sample number 1 which consisted of DNA extracted from vegetal feed, probably without undergoing to so much manufacturing processes it can be noticed that even if the concentration, as revealed by the spectrophotometric analysis, is increased (Table 2) and the quality ratio are in the accepted ranges, the pattern in the gel shows that a large part of the DNA is fragmented into smaller pieces that appear as a smear in the gel (Fig. 1). However, compared with the other analyzed samples we can conclude that this one is a good DNA sample in terms of quality and quantity. But, the case of sample 1 is a god example for arguing whatever it is necessary to use the both methods when the accuracy of determinations is very important. In order to be used further for amplification reaction the DNA solution containing this quantity, must be diluted. But after having

the image of the DNA solution on an agarose gel it is clear that, since most of the genomic DNA is degraded the dilutions must not be very severe. In the case of sample number 2, consisting of DNA extracted from an different vegetal feed, the electrophoretic pattern is resembling with the previous one, but they are very different in terms of concentration. Here a small amount of degraded DNA can be observed (Fig. 1).

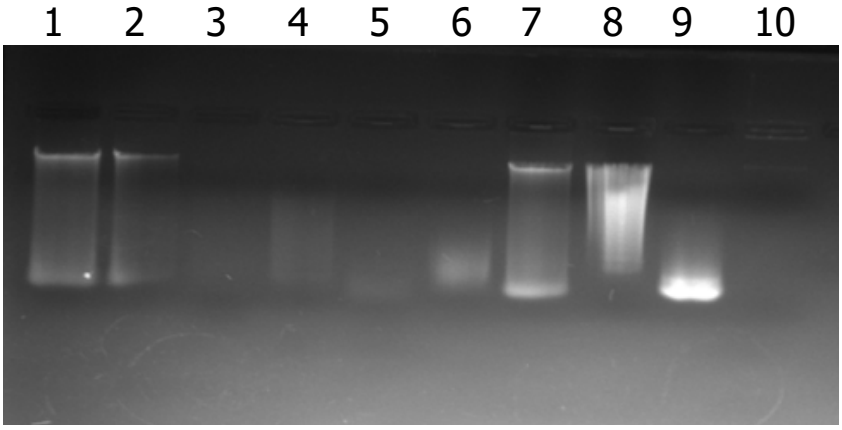

Fig. 1. Agarose gel electrophoresis of analyzed samples

Samples 3, 4, 5, 6 were found to have very low quantities of DNA, and from the analysis of agarose gel it can be noticed that those DNA solutions are totally fragmented. However, in this case, the DNA can be used in biochemical analysis, if the aim is to study a reduced DNA target. The sample 7, DNA isolated from pet food, has a similar pattern as in the case of sample 1 and 2. As for sample 8, calf muscle tissue, there is a high amount or DNA but the percentage of fragmented DNA is also very increased. The DNA isolated from liver, is in very high amount, but also in totality fragmented. Interestingly, here the fragments size seems to be constant since there is no visible smear for this sample. Sample number 10, consisted of DNA isolated from corn flour, which was previously diluted to an amount of 25 ng/µl. This concentration was confirmed by spectrophotometric analysis and the good quality can also be noticed from the agarose gel. In this case the genomic DNA appears as a single, compact band in the superior part of the image. Due to the low quantity the DNA band is hardly visible.

By conducting this study, it was possible to subtract some conclusion concerning the usage of the two presented techniques. Therefore, in this framework, for a better evaluation of DNA quality each of the analyzed methods are presenting some characteristics. By evaluating the spectrophotometric method some advantages were noticed, such as: is a easy to use and to learn method,

also it can give accurate results regarding the quantity of nucleic acids that are present in the analyzed solution. Moreover, the UV- VIS technique is non-destructive to the sample and has a high sensitivity for detecting organic compound as it was suggested by bibliographical references (3, 4), that may act as enzyme inhibitors. In the case were high performance spectrophotometers are used, also the time required for analyses is reduced since there is no need of performing dilutions and also, small volumes of sample are required for the analyses. Among the disadvantages of this method a highlight should be given to the fact that is not informative concerning the degree of DNA fragmentation in the solution and the result are depending very much of some physical factors such as the temperature.

By comparison, according to the description of Westermeier, 2004, the electrophoresis method has own characteristics such as: it is the best method that can be used in determining the DNA fragmentation degree, it can also be used in interpreting the amount of nucleic acids but beside special reagents for that analysis software's are required (9). Also, is a laborious technique and require a certain level of expertise for the user. It can also be expensive because, apart from the spectrophotometric method, for performing a nucleic acid electrophoresis, reagents such as agarose, DNA dyes and buffers are necessary and the volume of used sample is considerably higher than in the previous case.

Conclusion

High quality, intact pure DNA is required for many applications. Care must be taken to ensure reliable and reproducible results. Using poor quality DNA or DNA of unknown concentration can lead to problems during analysis leading to inaccurate results.

If sample quality checks are not done properly it is still sometimes possible to identify DNA quality issues after the enzymatic reactions, however this is not guaranteed. It is likely that problems with sample quality will only be identified after running the enzymatic reactions, at which point significant investment in time and money will have been made.

Acknowledgements

This work was conducted in Laboratories A1c and A2 from Horia Cernescu Research Unit of Banat's University of Agricultural Sciences and Veterinary Medicine „ King Michael I of Romania", Timisoara, Romania.

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CORRELATION BETWEEN CLINICAL SIGNS AND RADIOLOGICAL IMAGING IN PLEURO-PULMONAR AFFECTION IN DOGS

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Summary

The aim of the paper was to make a correlation between clinical signs and the aspect of thoracic cavity on the radiographic or CT imaging. The study was conducted on a number of 67 dogs of different age and sex, that were examined for thoracic pathology in the Faculty of Veterinary Medicine Cluj Napoca clinic. The patients were clinically examined and medical imaging procedures were performed in order to establish a diagnostic. From the total of 67 cases, 14% (N=9) were diagnosticated with pneumothorax, 18% (N=12) presented liquidothorax, 31% (N=21) were diagnosticated with inflammatory pathology of the lungs, 9% (N=6) present pneumonia abingestis, 7% (N=5) were diagnosticated with pleural pathology and 21% (N=14) diagnosticated with tumoral pathology. At the same time, we intend to identify the recommendations and limits of the radiological (radiography and Computed Tomography) examination in these conditions in dogs.

Keywords: thorax imaging, thorax pathology, lung imaging, dog

Evolution of technology produce a tremendous development of the diagnostic procedures especially in the field of medical imaging. Due to lung parenchyma, bronchia's and other thoracic structures, radiographic evaluation of the thorax has a high degree of difficulty. Radiography and Computed Tomography (CT) being the gold standard in diagnostic of thoracic pathology.

The radiographic examination of the thorax requires at least two exposure, one exposure being done with the patient in latero-lateral recumbency, left or right, and a second exposure is done with the patient in dorso-ventral recumbency (1).

Radiological approach to the lung requires following some steps:

- assessment of the degree of opacity of the lung;
- assessment of the bronchial, interstitial and alveolar aspect;
- the identification of hypo vascularization or hyper vascularization;
- identification of the affected area (cranio-ventral or cranio-dorsal, localization at a certain area of the lung, etc.;
- degree of severity of lesions, identification of the bronchial, alveolar, interstitial (nodular, nonspecific), vascular, mixed pattern (2, 3), degree of change in pulmonary pattern, differential diagnosis according to lesion localization.

These data should be correlated with the age of the animal, anamnesis, clinical examination and laboratory analysis to establish a correct diagnosis.

To obtain quality images, the radiography must be taken during inspiration, when the thoracic distension is maximum. Artificial ventilation can be attempted. It is advisable to make 3 exposures: a right-side lateral exposure; a left-side lateral exposure and a ventro-dorsal exposure. If the animal suffers from severe dyspnea, it is possible to perform radiography with the patient standing, as indicated by the cardiac examination. To reduce the possibility of blurry images due to respiratory movements, kilovoltage will be increased and exposure time and milliamperage will be reduced (4). Symptomatology that requiring radiological examination of the thorax includes: coughing, dyspnea, tachypnoea, bradypnea, tumorous conditions, trauma, exercise intolerance, weight loss.

Materials and methods

The study was conducted on a number of 67 dogs of different age and sex that were examined for thoracic pathology in the Faculty of Veterinary Medicine Cluj Napoca clinic. The patients were clinically examined and medical imaging procedures were performed in order to establish a diagnostic.

From the total of 67 cases, 14% (N=9) were diagnosticated with pneumothorax, 18% (N=12) presented liquidothorax, 31% (N=21) were diagnosticated with inflammatory pathology of the lungs, 9% (N=6) present pneumonia abingestis, 7% (N=5) were diagnosticated with pleural pathology and 21% (N=14) diagnosticated with tumoral pathology.

For each patient a thorough clinical examination was conducted, identifying the main symptoms, followed by a radiographic, Computed Tomography (CT) examination or both.

The radiographic examination was performed using a TEMCO GRX-01 roentgen device (producer K&S Rontgewerk Bochum-Germania), the images were acquired on a DR system. The CT examination was performed with patient under sedation, on a Siemens Somatom Scope system (producer Siemens).

The aim of the paper was to make a correlation between clinical signs and the aspect of thoracic cavity on the radiographic or CT imaging.

At the same time, we intend to identify the recommendations and limits of the radiological (radiography and Computed Tomography) examination in these conditions in dogs.

Results and discussions

On the clinical examination the most common symptoms present in all the patients was dyspnea, followed by tachypnea (in 47 cases), coughing (51 cases), loss of appetite (23 cases), decrease tolerance to effort (19 cases), vomiting (2 cases), apatia (21 cases).

For the radiographic examination we have opted for three exposure of the thorax. That was require because the lesions located in the lung parenchyma will be masked if the lung is collapsed due to the other lung weight. On the lateral radiography is evident the lung that is away from the table, being filled with air.

In 14% (N=9) of the patient diagnosticated with pneumothorax the symptoms were loss of appetite, loss of weight and moderate dyspnea. The radiography is enough to indicate presence of free air in the thorax cavity (Fig 1). Base on the radiography is hard to evaluate the degree of lung injury and the quantity of free air present in the thorax.

The CT examination is best in case of pneumothorax, being able to offer data about the lung status and indicate the lesions that produce the pneumothorax in the first place (Fig. 2, 3).

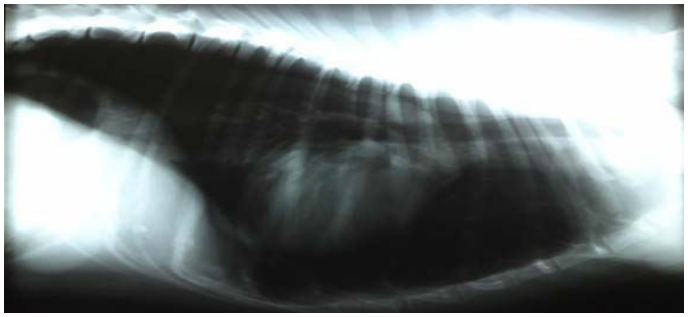

Fig. 1. Lateral radiography of the thorax, free air present in the thorax cavity

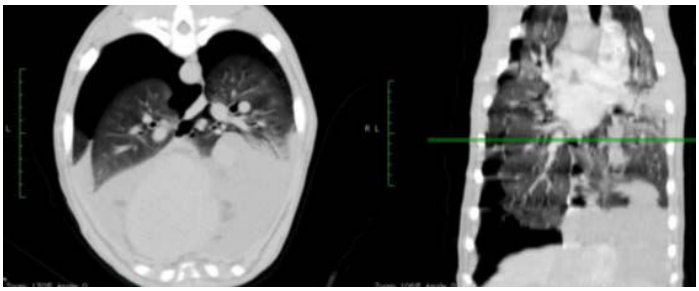

Fig. 2. CT of the thorax, lung window, pneumothorax and lung collapse

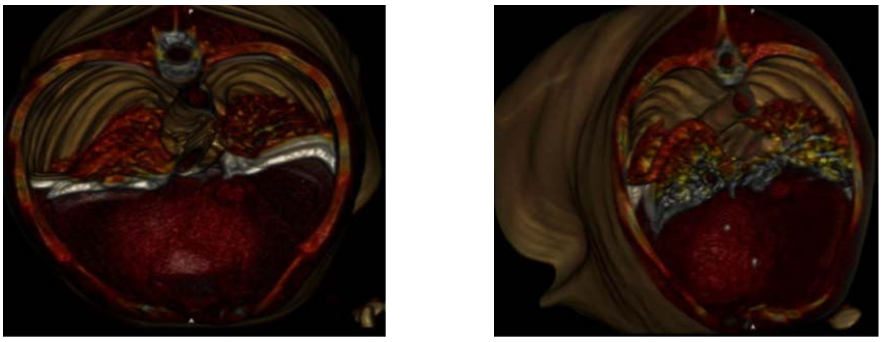

Fig. 3. VRT reconstruction of the thorax cavity with pneumothorax

In the 18% (N=12) of the patient diagnosticated with liquidothorax the symptoms consist of severe dyspnea, tachypnea, reduce effort capacity. The radiography help indicates the liquid in the thorax (Fig. 4), but have reduce capacity to identify a possible mass or other kind of formation if it's covered by the liquid (Fig. 5).

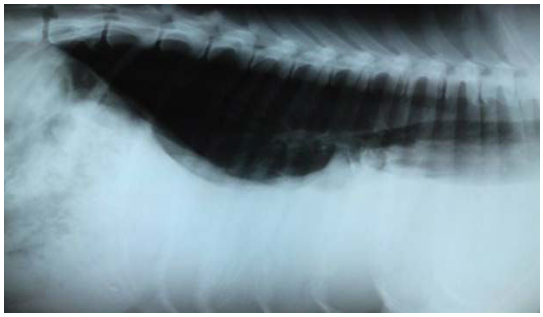

Fig. 4. Liquid thorax in dog, latero-lateral exposure

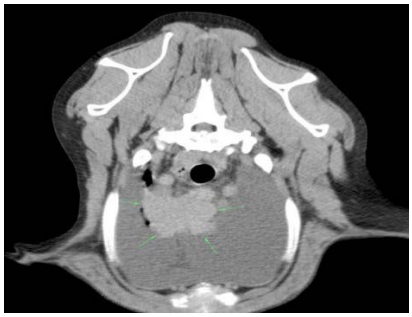

Fig. 5. CT of the thorax, mass in the mediastinum masked by the fluid in the thorax

For the 31% (N=21) that were diagnosticated with inflammatory pathology of the lungs, for the 9% (N=6) that present pneumonia abingestis (Fig. 6), and the 7% (N=5) that were diagnosticated with pleural pathology the symptoms consist of dyspnea, coughing, loss of appetite. The radiography shows changes of the pulmonary patter being able to indicate the degree of inflammation (Fig. 7). The CT examination can show subtler lesion of the pleura and the lungs (Fig. 8).

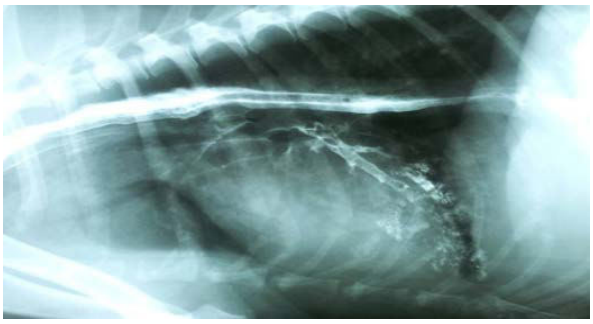

Fig. 6. Lateral radiography of the thorax, presence of contrast agent in the main bronchia, pneumonia abingestis

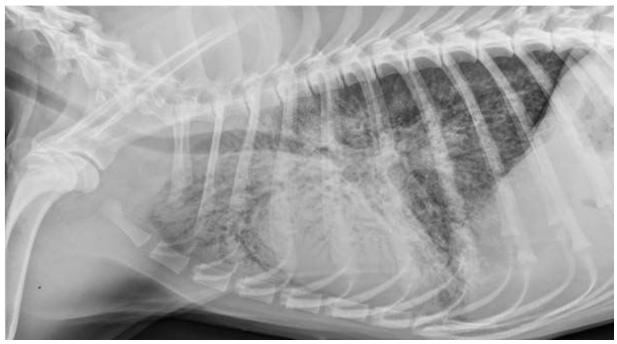

Fig. 7. Radiography of the lung, lateral exposure, pneumonia

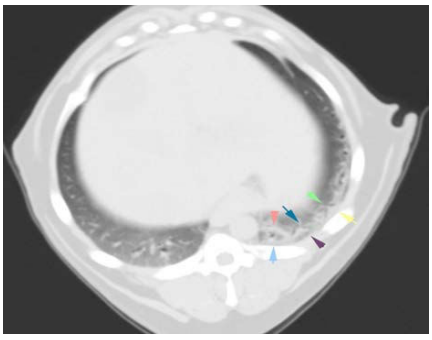

Fig. 8. CT of the thorax, consolidation of the lung and ground glass opacity

In the case of the 21% (N=14) diagnosticated with tumoral pathology, the symptomatology was mixt, varying from no respiratory symptoms to dyspnea and weight loss. The radiography can show a severe nodular pattern but one can miss subtle nodular formation or diffuse changes of the lung due to lung metastasis (Fig. 9, 10).

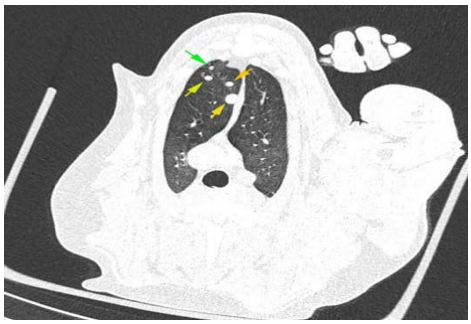

Fig. 9. Small nodular pattern in the lungs, metastasis from a primary bone tumor

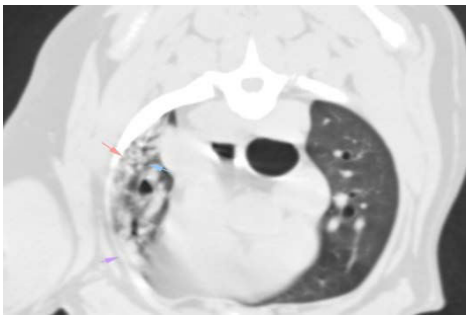

Fig. 10. Tumoral infiltration of the lugs due to primary bone tumor

Conclusion

The radiographic examination is a mandatory part of the thorax evaluation. The symptomatology found in the lung pathology vary amongst the patients, and is not a criterion for establishing a diagnostic. The radiographic examination has to include 3 exposure in order to offer the information necessary for a diagnostic. Even if the radiography is at hand the golden diagnostic method for thorax and lung pathology remain the Computed Tomography.

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MULTIBACTERIAL INFECTION IN RED SQUIRREL (*SCIURUS VULGARIS*) YOUNGSTERS: A CASE REPORT

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Summary

The objective of the study was to describe the results of both clinical and laboratory investigation performed for two clinical cases involving red squirrels (*Sciurus vulgaris*).The animals, male and female, aged approximately one month, were rescued on a university campus, in September, 2018. The youngsters showed no signs of disease for one week, when clinical examination revealed loss of appetite and weight, loose and watery feces, the presence of crusty nodules disseminated on the entire body, hair loss, edema of the ears, pads, anus, penis and vulva, respectively. Further in the course of the disease, necrosis enveloped the ear margins and haemorrhagic lesions appeared on the pads, anus and ears. Squirrelpox, a lethal viral disease, was suspected based on the clinical signs and lesional aspects. Both animals recovered after several days of supportive and symptomatic therapy. Laboratory evaluation also included microbiology and dermatology methods (selective media, biochemical properties evaluation, *in vitro* antimicrobial susceptibility testing) aimed to isolate and identify the associated dermatophytes and bacterial flora.

A diverse and complex skin flora was recovered from the skin lesions, with strains belonging to six genera and displaying distinct antimicrobial resistance patterns. To our knowledge, this is the first description of a mixt infection *in Sciurus vulgaris.*

Keywords: *Sciurus vulgaris*, necrotic lesions, associated bacterial flora, antimicrobial resistance

Red squirrels (*Sciurus vulgaris Linnaeus 1758*) are widely distributed throughout Eurasia, occupying various coniferous and mixed-deciduous forests (11, 25).In several European countries, the introduction of the non-native North American Eastern grey (*Sciurus carolinensis*) is increasingly recognized as a causefor the continuous decline in the population of *Sciurus vulgaris* (3, 5, 7, 8, 9, 15, 17, 23, 24, 25). Grey squirrels compete with the smaller rodents for resources and usually are asymptomatical carriers of Squirrelpox virus (SQPV) (Family *Poxviridae*, Subfamily *Chordopoxviridae*), which causes a fatal disease in most of the red squirrels(6, 13, 19). Squirrelpox is characterized by erythematous exudative dermatitis, with haemorraghic crusts, primarily on the lips, nose, eyelids, medial areas of the legs, toes, and ventral skin of thebody (5, 6, 7). The red squirrel is a major UK conservationconcern and understanding its continuing decline is important for any attempt to mitigate the population decrease (7, 16). Most fatalities are assigned to traffic-related injuries, predators, malnutrition, Squirrelpox and other infectious diseases (15, 18, 20, 22). Few cases of bacterial (4, 6, 10, 12, 14,

17, 22, 23,) or fungal (14, 21) infections have been documented in Europe and none, to our knowledge, in Romania.

Materials and methods

The study was conducted on two European red squirrels, male and female, evaluated to be approximately one month of age. The youngsters were found on the Agricultural Sciences and Veterinary Medicine University campus, Cluj-Napoca, having fallen, presumably, off the same nest. The subjects underwent sampling from various sites of the body, related to the location of the lesions: ear, skin, interdigital, genital and perianal area. The swabs were subjected to cultivation on Nutrient Agar (Tulip Diagnostics, Verna, India), then transferred onto MacConkey Agar (Tulip Diagnostics, Verna, India), Brilliance E. coli/Coliform selective medium (Oxoid, Hampshire, England), Chromogenic UTI medium (Oxoid, Hampshire, England) and Chapman Stone Agar (Himedia, Mumbar, India). Aerobic incubation was done at 37°C for 24h to 48h. Skin scrapings and plucked hairs were inoculated on D.T.M. Agar Base(Himedia, Mumbar, India) and kept for three weeks at room temperature, for the isolation of dermatophytes. The cultural characteristics of bacterial and fungal populations were interpreted based on colony morphology, color production and growth time. Microscopic assessment was based on bacterial morphology, arrangement and Gram staining. Pure bacterial cultures were obtained and transferred onto blood agar (Blood Agar Base - Himedia, Mumbar, India) with addition of 5% sterile defibrinated blood, in order to be further subjected to identification by Vitek®2 equipment, using VITEK® 2 analyzer, GN ID Cards (Gram-negative fermenting and non-fermenting bacilli) and VITEK® 2 GP ID Cards (Gram-positive cocci and non-spore forming bacilli). In the cases in which Vitek®2 identification was not possible, the examination was resumed to classical microbiological techniques. Antimicrobial susceptibility testing was performed according to the CLSI standards (2), by Kirby-Bauer disc diffusion method, against chloramphenicol, enrofloxacin, trimethoprim/sulfametoxazole, amikacin, ampicillin, amoxicillin/clavulanic acid, gentamicin and colistin test discs (Bioanalyse, Turkey).

Results and discussions

The squirrel youngsters were fed with cat milk substitutes for the following five weeks after being recovered. No signs of disease were registered for the first week, when clinical examination revealed loss of appetite and weight, loose and watery feces and unilateral otitis. Crusty nodules appeared on the lids, ears and on the dorsal sacral region, disseminating on the entire body over another week. Edema of the ears, pads, anus and genitals was seen shortly after, accompanied by exudation and hair loss in the lesional sites (Fig. 1). Further in the course of the disease, necrosis enveloped the ear pinnae (Fig. 2) and, in the case of the male squirrel, hemorrhagic lesions occurred on the pads, anus and ears.

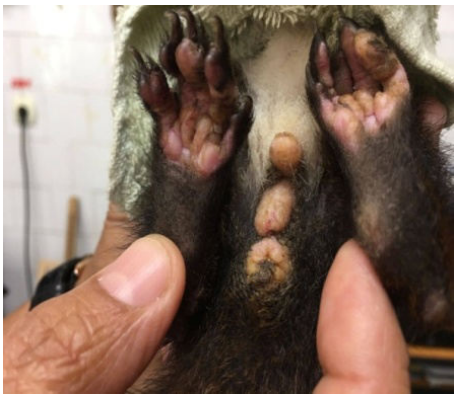

Fig. 1. Clinical aspects – edema, exudation and crusting of the pads, penal, scrotal and anal area

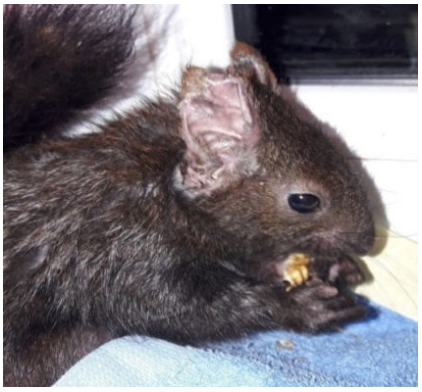

Fig. 2. Clinical aspect – necrosis of the ear pinnae in the third week of disease (female)

Due to the described lesions, Squirrelpox was suspected. Although typically fatal, if presented in the early stages of the disease, recovery chances are increased (6, 18), hence a therapy protocol was designed. Swabs were taken prior to any treatment and medication was implied as soon as clinical symptoms were apparent. Acknowledging that secondary infection can complicate the course of the viral disease, additionally to anti-inflamatory medication – meloxicam (1 mg/kg PO q12hr x 5 days), the youngsters received antimicrobial treatment - trimethoprim/sulphametoxazole (15mg/kg PO q12hr x 7 days) (1). A slight improvement, i.e. increase of appetite and energy was noted in both squirrels, approximately four days into the treatment. Complications appeared only in the male, a week after the treatment was completed. Edema aggravated and hemorrhagic crevasses occurred mostly on the toes, pads and anus. The decision was made to administer amikacin (3mg/kg IM q12hr x 7 days), based on antimicrobial susceptibility test results. The edema subsided and the general health status improved after three administrations of the antibiotic. Nonetheless, lesions expanded in both patients on the ear pinnae and tail skin (ventral side), with prominent crusting, necrosis and hair loss. Based on the alopecic appearance of the lesions, dermatophytosis was suspected. Samples were inoculated on D.T.M. Withinsix days, white, fluffy colonies appeared, with reddening of the medium. Microscopic examination revealed the presence of fusiform, solitary macroconidia, with few microconidia, consistent with the genus *Microsporum*. Topic administration of fentilconazole (once a day) proved efficient and lesions mended over the course of approximately one month.

Various populations of microorganisms were isolated and pure bacterial cultures were obtained in order to be further subjected to Vitek identification based on the measurement of several metabolic activities of microorganisms. *Bacillus*

licheniformis and *Lactobacillus spp*. were identified using standard microbiological investigations (SMIs). A total of six genera and eight species of bacteria could be identified (Table 1).

Table 1

Bacterial isolates from various anatomical regions

Subject	Sampling site	Organism
Male	Ear	*Enterobacter cloacae* *Enterococcus faecalis*
	Skin (dorsal region)	*Enterobacter cloacae* *Staphylococcus warneri*
	Interdigital	*Pseudomonas aeruginosa*
	Genital	-
	Perianal	*Pseudomonas aeruginosa* *Enterococcus faecalis*
Female	Ear	*Staphylococcus lugdunensis* *Bacillus licheniformis*
	Skin (dorsal region)	*Staphylococcus epidermis*
	Interdigital	*Staphylococcus warneri* *Pseudomonas aeruginosa*
	Genital	*Enterobacter cloacae*
	Perianal	*Staphylococcus lugdunensis* *Lactobacillus* spp.

Few antibiotics have clear recommendation of administration in this species. In this concern, extrapolation had to be made from those with indication for other species of rodents (1). Although resistance to trimethoprim/sulfamethoxazole of *Pseudomonas aeruginosa* is well known, susceptibility testing included the antimicrobial, as it was implied in the therapy. Total resistance could be confirmed. *Staphylococcus lugdunensis, Staphylococcus epidermis, Staphylococcus warneri* isolated from the female squirrel proved to be the most resistant bacteria. The MAR (multiple antibiotic resistance) index applied to a single isolate was 0.44 for all three microorganisms. Contrary to expectations, the strain of *Pseudomonas aeruginosa*, although represented by a MAR index of 0.22, did not prove the most resistant. Moreover, bacterial isolates from the female squirrel comprised a number of 7 species, whereas flora identified on the male subject consisted of only 4 species.

Conclusions

The rich population of bacteria isolated and identified, most of which showing multiple resistance to antibiotics certifies the presence of bacterial infection and raises concern in the matter of antibioresistance, as it shows even in youngsters of *Sciurus vulgaris* which had never received any kind of treatment. Additionally, dermatphytosis complicated the course of the pathological processes and significantly expanded the recovery period. Considering that limited information is available concerning microbiota in *Sciurus vulgaris* worldwide, extended studies prove urgent, especially in countries where the species is suffering a striking decline. Further studies will be made on samples recovered from the two cases, in order to exclude the presence of Squirrelpox virus.

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MORPHOPATHOLOGICAL SIGNALING OF PNEUMONIA CAUSED BY BOVINE RESPIRATORY SYNCYTIAL VIRUS IN CALVES FROM TIMIS COUNTY

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Summary

The research was conducted in the period February 2017-June 2018, by performing necropsies on 10 calf corpses, aged 4-15 months, both male and female, of various breeds (mainly Romanian Black Spotted breed). The animals came from 2 farms (one is a collecting center farm from Grabat) and from households in Timis county, from owners, who solicited necropsies at the Forensics and Necropsy Diagnosis Department of the Faculty of Veterinary Medicine in Timisoara, in order to clarify the cause of death. Upon necropsy, the animals showed characteristic signs of lobular catarrhal pneumonia. Samples (tissue fragments of 2/1.5 cm) were collected, following detailed macroscopic examination of the organs, from the: lungs, heart, kidneys, liver, tracheobronchial lymph nodes and spleen, for microscopic exams.The pathological morphology picture of the cases subjected to morphopathological exams that certify the diagnosis of Pneumonia caused by bovine respiratory syncytial virus are expressed by: serohemorrhagic and fibrinohemorrhagic bronchitis and tracheitis, lymphohistiocytic bronchopneumonia, obstructive necrotizing, hyperplasic, polyp-like and syncytial bronchiolitis, desquamating, syncytial alveolitis, interstitial and alveolar emphysema with a tendency of extension in the entire pulmonary parenchyma (panlobular emphysema) and pulmonary atelectasis. The circulatory, dystrophic, and inflammatory cardiac, hepatic plenic, renal and lymphonodular lesions are non-specific.

Keywords:pneumonia, respiratory syncytial virus, calves

Pneumonia caused by bovine respiratory syncytial virus is an infectious, contagious, endemic disease seen in cattle. It is caused by a virus belonging to the *Paramyxoviridae* family, *Pneumoviridae* family, *Pneumovirus* genus, morphologically similar to the human virus (HRSV) and mice pneumonia virus. The bovine syncytial virus is closely related to the human syncytial virus (13, 6, 3). The two viruses have common epidemiological, clinical and anatomopathological characteristics.

The protein structure of the bovine strains and human strains is similar, with a minor difference, namely the molecular weight. The syncytial respiratory viruses (human and bovine) are the most frequent cause of lower respiratory tract infections. In humans, the virus has been isolated from the nasopharynx of apparently healthy children (Isaia et al. 1985, cit. Paul, 2012) (8).

Bovines under 18 months of age are most frequently affected while sheep

and goats are rarely affected. The disease has an enzootic character and acute evolution and it is clinically expressed through respiratory disorders [30/?]. Morphopathological signs include pulmonary emphysema accompanied by atelectasis and bronchopneumonia. Bovines are the receptive species, with maximum sensitivity recorded in animals under the age of 3-9 months. Most animals are affected for the first time at the age of 1-12 months (6, 8, 11). The virus enters the organism via the respiratory path and multiplies in the cells of the nasal, pharyngeal, tracheal, bronchiolar epithelium and in alveolar pneumocytes type I and II. The virus is also present in alveolar macrophages (1, 12, 13).

The first signaling in bovines date back to the year 1967 when the virus was detected in Japan, Belgium and Switzerland. Later, it was also detected in the UK and USA. The infection is spread worldwide and is of great economic importance due to high morbidity rates, decrease of weight gain and deaths (9, 13).

Materialsandmethods

The research has been conducted in the period February 2017-June 2018. Necropsy exams were performed on 10 corpses from calves aged 4 months to 15 months, both M and F, of different breeds (mostly Black Spotted), from two farms- one being a collecting center farm from Grabat and from private households in Timis County, from owners that have solicited a necropsy exam at the Forensics Department of the Veterinary Medicine Faculty in Timisoara. The aim of the exams was to clarify the cause of death. Upon necropsy, the animals showed characteristic signs of lobular catarrhal pneumonia. Samples were collected (2/1.5 cm tissue fragments), following a detailed macroscopic examination of the organs, from: lungs, heart, kidneys, liver, tracheobronchial lymph nodes and spleen, for microscopic examination. The samples were fixated using 10% formaldehyde, for 24 hours. The obtained block were sectioned using a microtome, at 6 micrometers from each block and placing 2-4 sections on the slide. The sections were stained sing the trichrome method- Haematoxylin- Eosin- Methylene blue to enhance the modified structures (7).

After staining, the sections were dehydrated and mounted in an anhydrous environment using Canada balm. The histopathological preparations were examined using an Olympus CX41 microscope (acquired through POS CCE, DICES-MVT 2669-145), with increasing objectives. They were then interpreted and microphotographed.

Resultsanddiscussions

External examination.The calf corpses showed a mediocre towards good condition, with apparent mucosae that were cyanotic and moist (Fig. 1). In three cases, the subcutaneous conjunctive tissue from the cervical area showed gas bubbles (subcutaneous emphysema) and five cases presented serous exudate (subcutaneous oedema), enlarged retropharyngeal lymph nodes, with sections

colored in red alternating with grey-whitish areas (focal haemorrhagic lymphadenitis).

Fig. 1. Calf corpse, mediocre condition with cyanotic and moist apparent mucosae

Internal examination.In the lumen of the trachea and bronchi, all cases had a foamy fluid, with blood and after the removal, the mucosa was red-coloration maintained after washing- serohemorrhagictracheobronchitis.

Microscopically, there are signs of tracheal epithelium desquamation-epithelium is fallen into the lumen and is mixed with fibrinous haemorrhagic exudate-catarrhal-haemorrhagictracheitis and fibrinous- haemorrhagictracheitis (Fig. 2, 3).

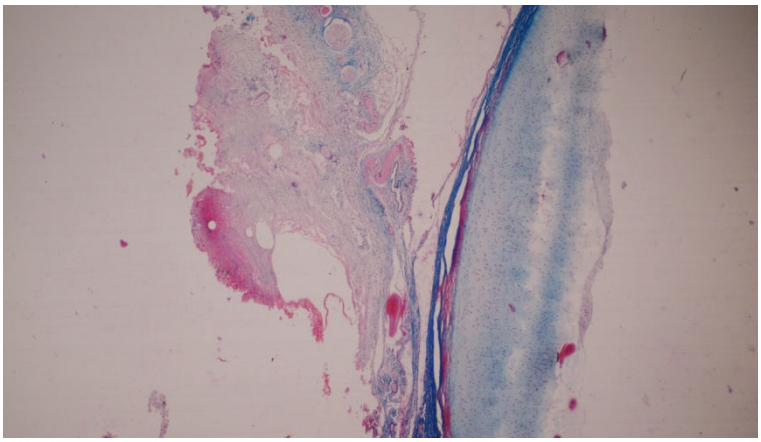

Fig. 2. Catarrhal-haemorrhagic transudate: haemorrhagic catarrhal exudate in the lumen of the trachea HEA staining x4

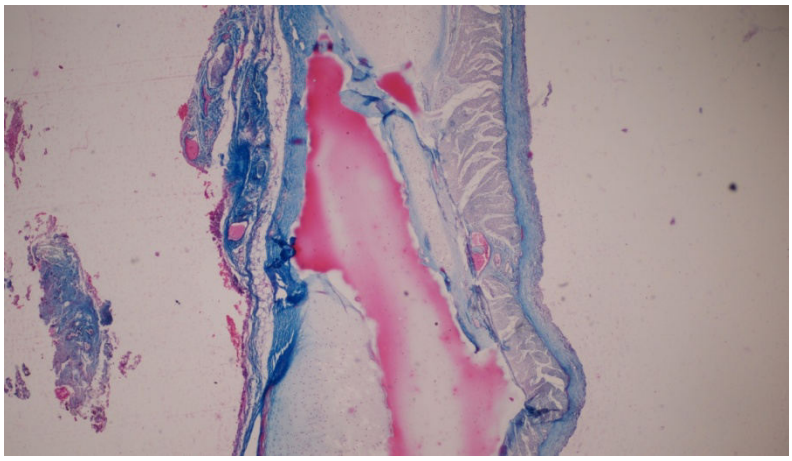

Fig. 3. Fibrinous-haemorrhagictracheitis: intra-luminal fibrinous-haemorrhagic exudate. HEA staining x4

Lungs

Macroscopically the lungs appeared enlarged in volume and weight, with a tense pleura and round edges. The cranial lobes (apical), middle (cardiac) and the upper-anterior 1/3 of the caudal lobes (diaphragmatic) had areas of pulmonary condensation, in various shades of red-from dark red to bright red, both on inspection and on section. The lung floatation test was positive- lymphohistiocytic interstitial bronchopneumonia, acute form (Fig. 4).

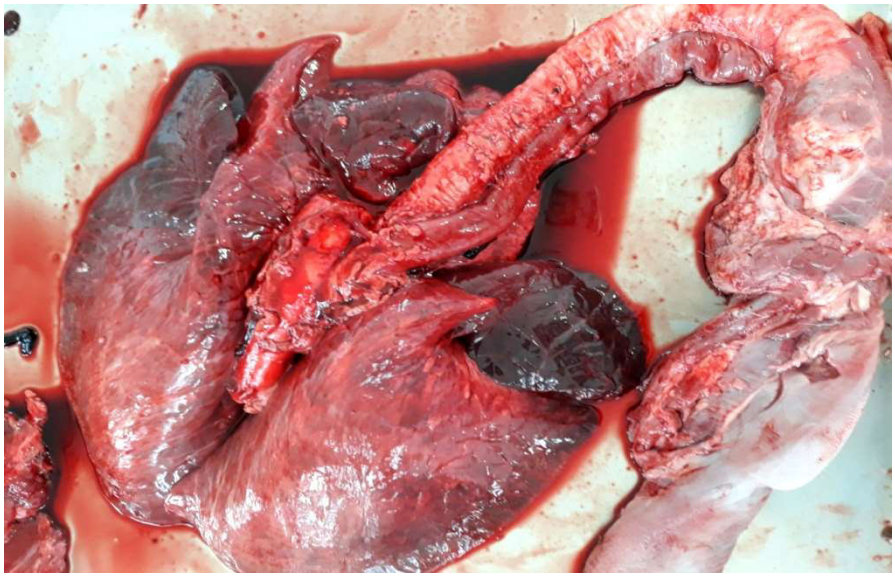

Fig. 4.Lymphohistiocytic bronchopneumonia, acute evolution

The diaphragmatic lobes, upper-dorsal area- pale pink color, different shades, numerous gas bubbles, crepitation consistency and negative lung-floatation test- pulmonary emphysema.

Microscopically, the following were visible:

- desquamation and syncytialization of pneumocytes (desquamation alveolitis) associated with lymphohistiocytic, inflammatory cellular infiltrate in the alveolar setae, peribronchial and perivascular areas'
- areas of alveolar emphysema around the compaction areas present throughout the pulmonary parenchyma, especially in the caudal lobe- catarrhal, desquamation, syncytialization (Fig. 5, 6) bronchopneumonia- a characteristic lesion of bovine syncytial virus pneumonia (2, 4, 5, 8, 13).
- moderate metaplasia and hyperplasia of the bronchial epithelium, with the aspect of buds, enhanced by the transformation of the cubic epithelium into prismatic epithelium, with the formation of papilliferous or polyp-like prominences that greatly reduce the diameter of the bronchial lumen;
- desquamation accompanied by necrosis of the bronchial cells epithelium- after HEA staining has a dull, anhistic, astructural aspect, peribronchial and intraluminal alveolar emphysema- obstructive necrotising bronchiolitis and alveolar emphysema (Fig. 7, 8);
- the obstructive necrotising bronchiolitis and alveolar emphysema are the consequence of the cytopathogenic effect of the virus on epithelial cells (6, 9, 13);

- vascular congestion and pulmonary emphysema- a volumetric penumopathy secondary to bronchiolitis, responsible for the acute respiratory syndrome that defines the condition (BRSV) (4, 8, 13);
- pulmonary atelectasis around the bronchopneumonia and emphysema areas-the latter being responsible for the accentuated decrease of the alveolar lumens due to compression (Fig. 9).

The alveolar emphysema is the consequence of the cytopathogenic action of the bovine, respiratory syncytial virus on the pneumocytes and on the fibroelastic component of the alveolar setae structure. The presence of the interstitial emphysema is explained by the structural particularities of the bovine lungs, namely the clear, lobular division that limits the interdependence between the adjacent lobes, creating thus, a collateral ventilation deficiency that favours the loss of air in the interstitium(3, 5, 8).

The syncytialization of the pneumocytes is achieved through their detachment from the alveolar setae, the merging of the cytoplasm, nuclei and cellular membranes compared to the syncytialization of the bronchiolar epithelium that happens through cellular hyperplasia processes-aspects similar to those seen in bovine parainfluenza (PI3) (8).

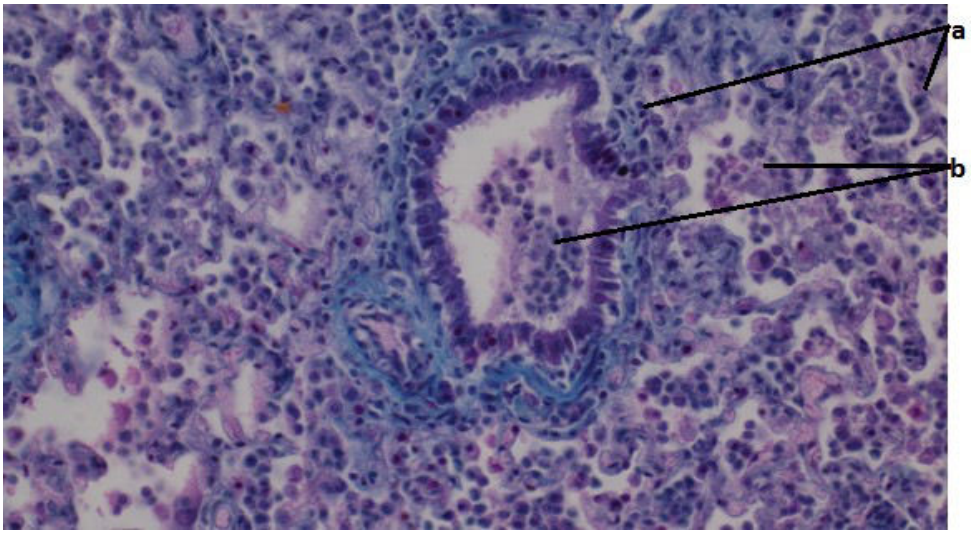

Fig. 5.Lymphohistiocytic bronchopneumonia peribronchiallymphohistiocytic hyperplasia and in the alveolar setae, desquamation and syncytialization of the intraalveolarpenumocytes; hyperplasia and syncytialization of the bronchial epithelium HEA staining x 40

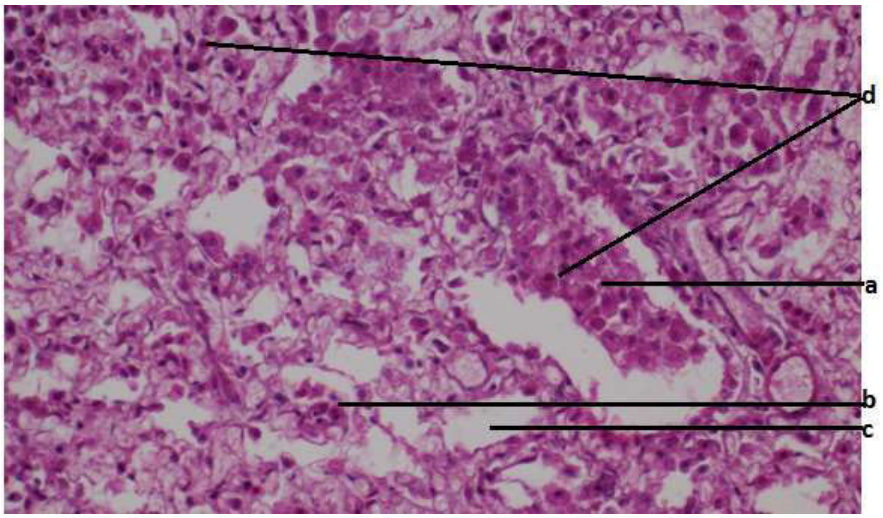

Fig. 6.Lymphohistiocytic bronchopneumonia: hyperplasia and polyp-like syncytialization of the bronchial epithelium, dequamativealveolitis and syncytialization (b); alveolar emphysema (c); oxyphile viral inclusions in the alveolar and bronchial epithelial cytoplasm (d). HEA staining x40

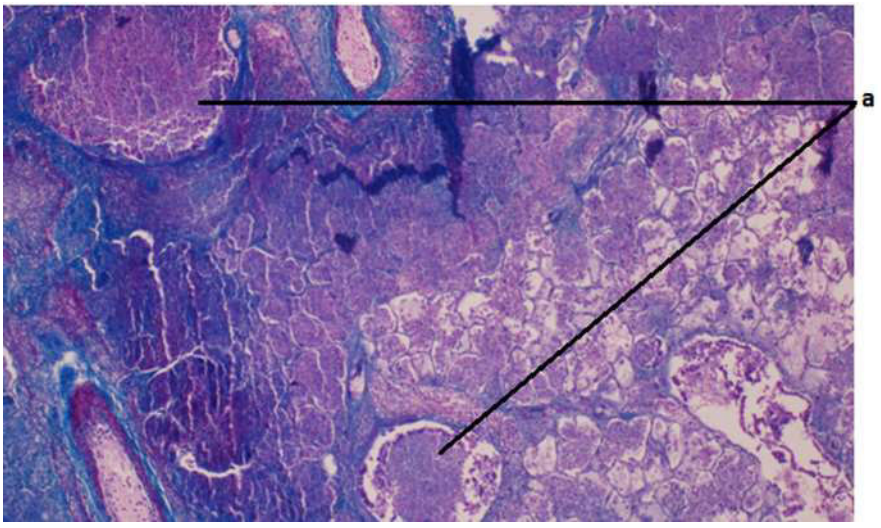

Fig. 7.Obstructive, necrotizing bronchiolitis, transversal section: bronchial epithelium dequamation and necrosis with intraluminal blockage (a). HEA staining x 10

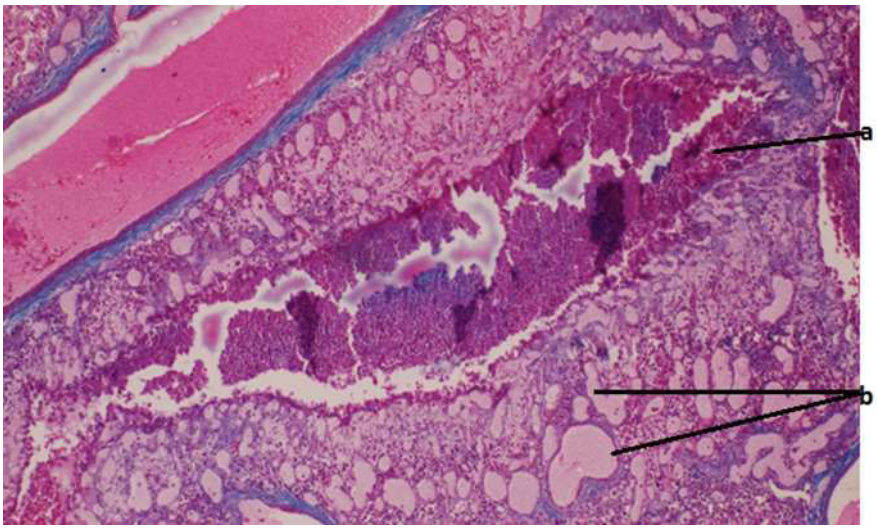

Fig. 8. Obstructive, necrotizing bronchiolitis and alveolar emphysema/longitudinal section: bronchial catarrh, necrosis and obstruction (a); peribronchial and intralobular alveolar emphysema (b). HEA staining x10

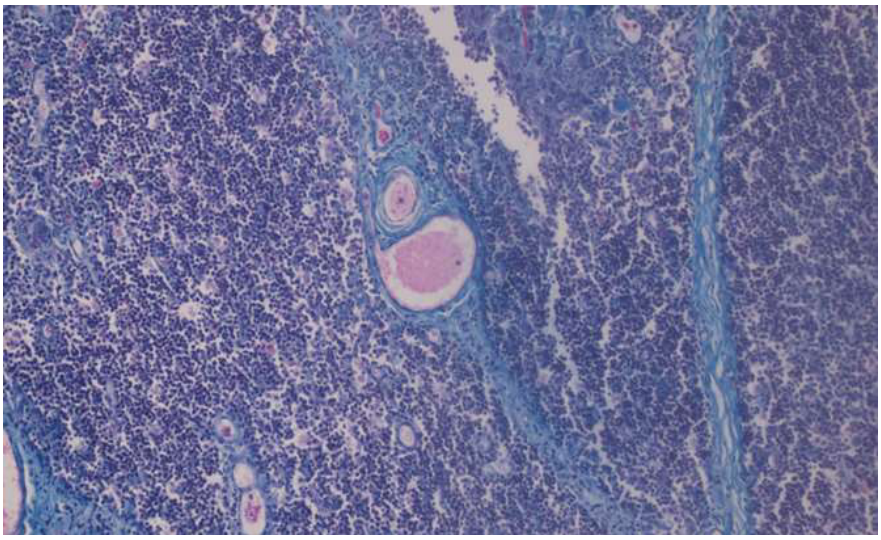

Fig. 9.Lymphohistiocytic bronchopneumonia: congestion and lobular atelectasis. HEA staining x 20

Heart

Macroscopically, on inspection and section the heart appears greyish, dull aspect, moist, without shine, aspect of boiled meat and friable consistency.

Microscopically, upon examination with x4, x10, x20, x40 objectives the following are visible: degenerate myocardiocytes, with numerous basophilic granulations, fine or gross, in the cytoplasm, hiding the nucleus and causing a turbid swelling aspect; ectasia and capillary haemorrhage, intraparenchymal and interstitial, subepicardialhaemorrhage (Fig. 10, 11).

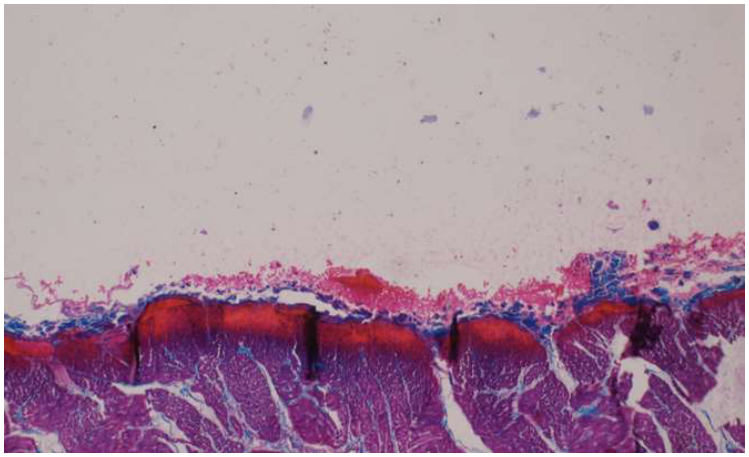

Fig. 10. Heart: subepicardialhaemorrhage. HEA staining x10

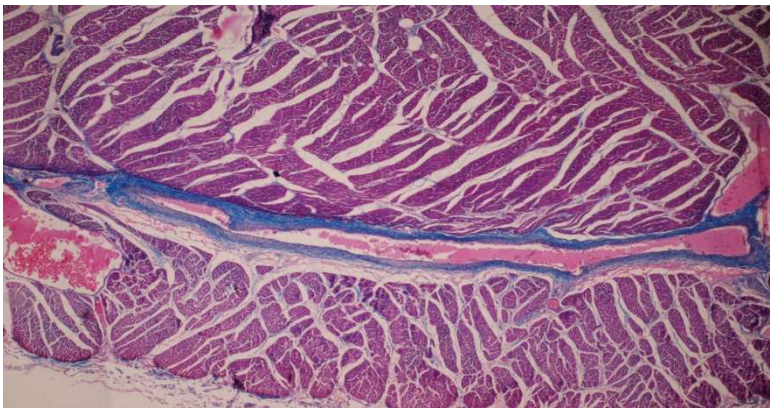

Fig. 11. Heart: intraparenchymal and interstitial haemorrhages HEA staining x4

Liver

Macroscopically: surface and section colours: yellowish-grey, shiny, greasy aspect, elastic consistency, physical-structural particularities that define a degenerative, inflammatory hepatopathy.

Microscopycally: in the cytoplasm of hepatocytes- fine granulations, gross and optically-empty vacuoles with and without implication of the nucleus; lymphohistiocytic plasmatic interstitial hyperplasia in the Disse spaces and perilobular areas- lymphohistiocytic plasmatic hepatitis (Fig. 12).

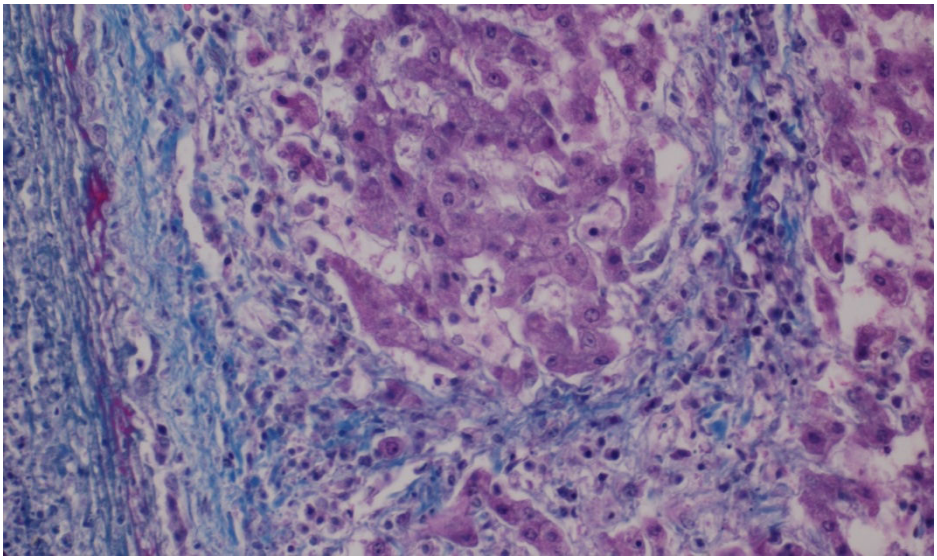

Fig. 12. Liver: perilobularlymphohistiocytic hyperplasia on a fine, conjunctive fibre network HEA staining x40

Kidneys

Macroscopically- they appeared enlarged in volume and weight, tense, transparent capsule, allows visualising of the parenchyma that appears grey-yellowish, dull, matte aspect. These modified physical-structural particularities are also present on section-protein nephrosis.

Microscopically- noticeable vascular modifications, of various intensities, translated through congestion, oedema and haemorrhages of small intensity in the cortical and medullar area; the epithelium of the renocytes detached and fell into the lumen of the uriniferous tubes, accompanied by the formaton of cellular cylinders; periglomerular and peritubular lymphohystiocytic hyperplasia-lymphohistiocytic nephritis (Fig. 13).

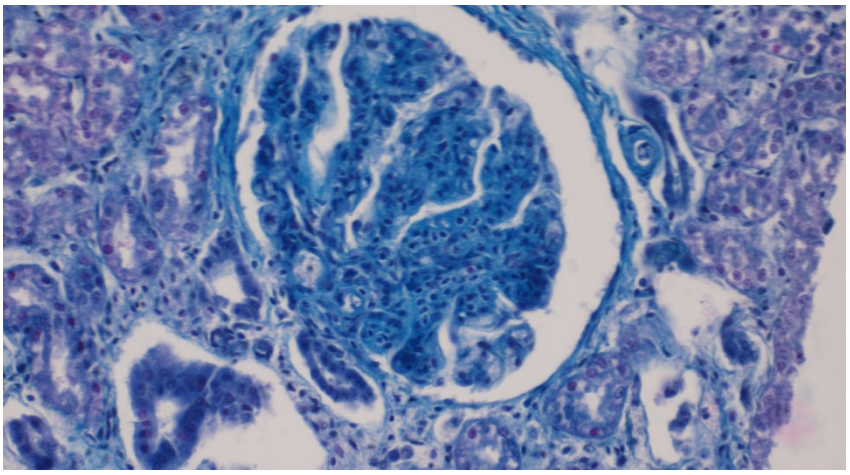

Fig. 13. Kidneys – lymphohistiocytic nephritis: periglomerular and peritubular lymphohistiocytic hyperplasia, urinary retention cysts, HEA staining x 20

Spleen

Macroscopically- enlarged in volume and weight, dark red color on inspection and section, absence of follicular pattern-pulp hyperplasia.

Microscopically: the red pulp is predominant and the white pulp is reduced by lymphocytoclastic processes and/or lymphocyte migrations, interstitial and perivascular fibrosis-pulp hyperplasia (Fig. 14).

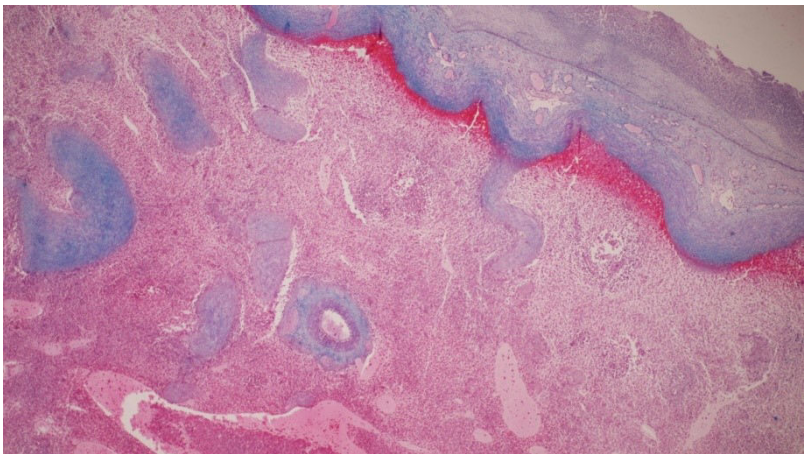

Fig. 14. Spleen – pulp hyperplasia: predominant red pulp, reduced lymph tissue due to lymphocyte migration and/or lymphocytolysis HEA staining x 4

Submaxillary, mediastinal and tracheobronchial lymph nodes

Macroscopically- enlarged, filled with fluid, elastic consistency; on section- mosaic aspect, grey-whitish areas alternated with dark red areas of various sizes and shapes: focal haemorrhagic lymphadenitis.

Microscopically- signs of subcapsular, peritrabecular and perifollicular haemorrhagic exudate, thrombosis of the central follicular capillaries, intraparenchymalhaematic lacunas: lymphocytoclastic processes and lymphocyte migrations- focal and diffuse haemorrhagic lymphadenitis (Fig. 15).

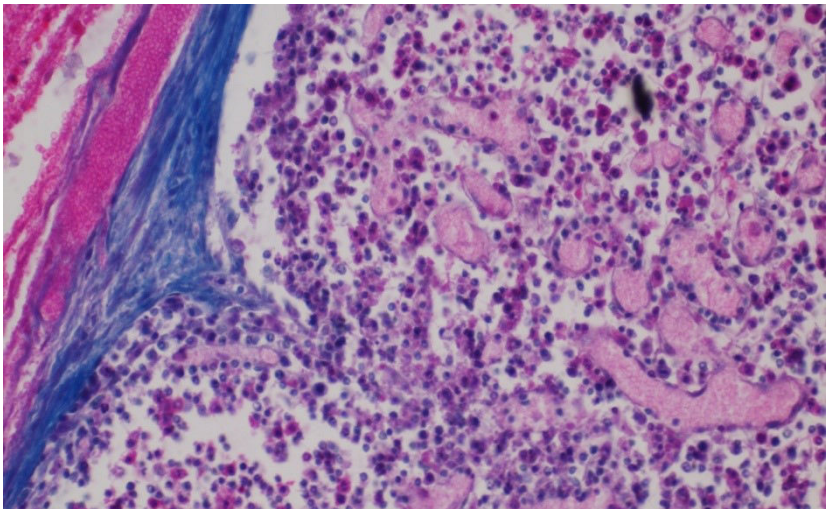

Fig. 15. Focal, haemorrhagic lymphadenitis: lacunar haemorrhagic exudate in the lymph node parenchyma HEA staining x 40

Conclusions

Pathomorphicpicture in the morphopathologically examined cases, which certifies the diagnosis of bovine respiratory syncytial virus pneumonia is expressed through: serohaemorrhagic and fibrinous-haemorrhagic bronchitis and tracheitis; lymphohistiocytic bronchopneumonia; obstructive, necrotizing bronchiolitis hyperplastic, polypus and syncytialization;desquamating and syncytializingalveolitis;alveolar and interstitial emphysema, with an extensive tendency troughout the pulmonary parenchyma (panlobular emphysema);pulmonary atelectasis.

The circulatory, dystrophic and inflammatory cardiac, hepatic, splenic, renal and lymph nodular modifications are non-specific lesions.

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EFFICACY ASSESSMENT OF THE AGAVAC VACCINE DESTINED FOR CONTAGIOUS AGALACTIA PROPHYLAXIS IN SMALL RUMINANTS, ON GUINEA PIG

SHORT COMMUNICATION

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Summary

In the aim of establishing an alternative model for efficacy assessment of the Agavac vaccine destined for the prophylaxis of contagious agalactia in small ruminants, the vaccine immunogenicity and the colonization ability of *Mycoplasma agalactiae* AG6 strain (Mag AG6) were tested on guinea pigs (common race, 300-450g). Guinea pigs, in groups of 3 animals, both females (pregnant or lactating), as well as males, were immunized with Agavac commercial series, by s.c. administration of two doses of 0.5 ml, at 21 days interval. At 4.5 months after the booster, the Mag AG6 strain, was administered in concentration of 0.8 mg/ml protein in the control animals (0.2 ml, by ocular instillation) or of 4.5 mg/ml protein in vaccinated animals (0.2 ml, by ocular or nasal instillation, or 1 ml , intramammary). Antibodies titers were evaluated by an *in house* made ELISA kit, and the presence of the Mag AG6 strain in the organism of the guinea pigs was detected by a rPCR test, based on the p40 gene, applied to samples/fingerprints of organ, milk and ocular and nasal secretions. The values of antibody levels, expressed in EU, during the experiments, recorded statistically significant differences ($p < 0.05$) between vaccinated and unvaccinated animals at all the testing moments. The strain Mag AG6, administered in vaccinated animals in concentration of 5.25 times higher than the dose administered to unvaccinated animals, was present at the inoculation site for up to 72 hours (in the case of the ocular or nasal instillation), as opposed to unvaccinated animals, in which it persisted until slaughter (13-14 days p. i). Our results proved that the assessment of the effectiveness of the Agavac vaccine can also be carried out on laboratory animals (guinea pigs).

Keywords: *Mycoplasma agalactiae* AG6, vaccine efficacy, rPCR, ELISA, guinea pig

Contagious Agalalactia (CA), an infectious disease of small ruminants is caused by several species of *Mycoplasma*, among which *Mycoplasma agalactiae* is constantly isolated (1,5). Disease prophylaxis focuses on the use of vaccines, in European Union only those inactivated being accepted (11). Agavac is an inactivated vaccine, manufactured by the Pasteur Institute for several decades, based on the *Mycoplasma agalactiae* AG6 strain (Mag AG6), isolated in 1951 in Romania, from an outbreak of CA in sheep. The registration, authorization / re-authorization of a vaccine requires the demonstration of its efficacy through immunological / serological data and the protection resulting from an experimental infection. Different models of experimental infections in the target species have

been published, with inoculation of the *M. agalactiae* vaccine / homologue strain by nasal, oral, ocular or intramammary route (3,4,6,7,8,9,10). In the aim of establishing an alternative model for efficacy assessment of the Agavac vaccine destined for the prophylaxis of CA in small ruminants, the vaccine immunogenicity and the colonization ability of Mag AG6 strain were tested on guinea pigs.

Materials and methods

Vaccine: commercial batches of Agavac vaccine, manufactured in 2015-2016, were tested in our experiments. For challenge, ***Mycoplasma agalactiae* AG6** was grown for 6 days under anaerobiosis condition, at 37^{o}C, on PPLO medium (Pasteur), supplemented with 15% horse serum and inoculated by the "running drop" method. The bacterial suspension used for challenge was made in PBS, pH 7.2±0.2 (Pasteur, NaCl 6.5g/L, Na2HPO4 x 12 H2O 5.6g/L, KH2PO4 0.9g/L). The uninoculated PPLO gelose Petri dishes, washed also by PBS, represented the "mock" sample for the guinea pigs control group. The quantification of protein concentrations for bacterial suspension and "mock" sample was performed with Qubit Protein Assay Kit (Thermo Fisher Sci., Q33211), according to manufacturer's instructions for use. **Animals**: There were used guinea pigs (Dunkin Hartley cross breed, 300-450g) in groups of 3 animals, both females (which were lactating or pregnant at the moment of challenge) and males. The animals were maintained in isolated cages, fed and watered according to the experimental animal house procedures. The experiments on animals have been approved under the laws and regulations in force concerning the protection of the animals uesd for scientific purposes. **Methods and experimental design: Vaccinated animals**: the guinea pigs were inoculated, by subcutaneous route, with 0.5 ml of Agavac vaccine, and a booster was performed (0.5 ml, vaccine) at 21 days post vaccination. **The control infection** was performed at 4.5 months after booster, with *Mycoplasma agalactiae* AG6 suspension of 0.8 mg / ml protein for unvaccinated animals, 0.2 ml / animal by ocular instillation and of 4.2 mg / ml concentration for vaccinated animals administered intra-mammary (1 ml by immersion of the mammary in bacterial culture), nasal or ocular instillation (0.2 ml, each route).**Serology**: The antibody titers, expressed as EU, have been determined by indirect ELISA, at 21 days after booster vaccination, at control infection time and at 14 days after challenge. An *in house* assay with antibody against guinea pig (Sigma Aldrich) was used (2). The guinea pig serum samples were diluted 1:100, and interpretation criteria were as follow: <30 UE negative, 30-35 UE suspect, >35 positive. **The presence of *Mycoplasma agalactiae*** in animals subjected to challenge was determined by rPCR technique based on p40 sequence, by processing organ fingerprints and secretions from subscapular lymph node, elbow joint, knee joint, testis or ovary, kidney, spleen, liver, heart, lung, brain, ocular, mammary gland, rectum, milk and blood (12). The organ fingerprints / secretion were taken on Dacron swabs which were then suspended in one ml of PBS (Pasteur). DNA extraction was performed

with the QIAamp DNA mini kit (Qiagen 51304) by adding 200 µl of AL buffer over 200 µl of the liquid sample followed by the manufacturer's protocol. The amplification was performed in volume of 25 µl (12.5 µl Briliant II Sybr Green qPCR Master Mix, Agilent 600828, 9.5 µl nuclease free water, 0.125 µl each primer, 0.75 µl Rox 1: 1000, 2 µl DNA) in Mx3005P amplifier (Stratagene / Agilent), according to the cycling program 50oC, 2 min - 1x + 95oC, 10 '- 1x + 95oC, 15' '+ 60oC, 1' - 50x + 60oC, 10 '+ 25oC, 1' - 1x , followed by the dissociation curve as is in the MxPro software, and in parallel, by electrophoresis in agarose gel (TBE1x), for the confirmation of the reaction (Mag AG6 generated Tm 75.85 - 76, with a 109 bp amplicon) (see Fig. 2 and Fig. 3). The detection limit of the amplification method, based on the p40 sequence previously established on the Mag AG6 strain, was 10 UFC / ml (data unshown). **Statistical analysis** of the data was performed by the t-Student test, with $p < 0.05$ statistically significant (MS Excel).

Results and discussions

Following the control infection, it was found that all animals, vaccinated or unvaccinated, survived, regardless of sex, physiological status, inoculum type / concentration and route of inoculation. No hyperthermia and nor prostration reactions have been recorded. In animals with *Mycoplasma agalactiae* AG6 administered by ocular instillation, discreet reactions of ocular hypersecretion were recorded. Serologically, ELISA revealed that at 4.5 months after booster vaccination, the antibody titers induced by Agavac vaccination showed a decrease statistically insignificant compared to the control at 21 days post - booster (Table 1 and Fig. 1). At 14 days after Mag AG6 inoculation, antibody titers in vaccinated and infected animals showed an increase, suggesting an activity similar to a booster vaccination. In the non-vaccinated animals, the antibody levels were in the negative area (less than 30 EU / ml) at all times of testing, including after administration of infectious innoculum. The titer differences among vaccinated and unvaccinated animals, were statistically significant ($p < 0.05$). Results obtained in rPCR assays regarding to the presence and persistence of *Mycoplasma agalactiae* AG6 in animals unvaccinated / vaccinated with Agavac atfer the infectious challenge are summarized in (Table 2 and Table 3). At 24 hours p.i. Mag AG6 was present in ocular secretion of the animals infected by ocular route, regardless of whether they were vaccinated or not. Also at 24 h p.i. Mag AG6 was present in milk samples from intra-mammary infected animals and at nasal level in nasal infected animals. During experiments, Mag AG6 was present at ocular level for maximum 72 hours in vaccinated animals, compared to unvaccinated animals, where it was present until the slaughter. Mag AG6 was detected also, at 14 days p.i in lymph node samples from animals vaccinated with Agavac and infected by nasal route, and in kidney samples from unvaccinated animals and infected by ocular route.

Table 1

The ELISA results on the anti-*Mycoplasma agalactiae* AG6 (Mag AG6) antibody titre expressed in the EU in the body of vaccinated / unvaccinated with Agavac and experimentally infected animals with Mag AG6

		Post-vaccination days		Post-infection days
Guinea-pig group	Experimental infection route / inoculum	21	135 (infection moment)	14
		ELISA Units (EU)		
Vaccinated 1 (Lactating)(V1L)	Intra-mammary (1 ml; 4.20 mg protein Mag/ml)	41 ±3	26 ±2	31 ±1
Vaccinated 2 (Gestating)(V2G)	Nasal instillation (0.2 ml; 4.20 mg protein Mag/ml)	71 ±5	55 ±4	86 ±4
Vaccinated 3 (Males)(V3M)	Ocular instillation (0.2 ml; 4.20 mg protein Mag/ml)	74 ±4	58 ±3	71 ±4
Unvaccinated 1 (Males)(UV1M)	Ocular instillation 0.2 ml (0.8 mg protein Mag/ml)	ND	12 ±2	3 ±1
Unvaccinated 2 (Males)(UV2M)	Ocular instillation 0.2 ml (0.8 mg protein Mag)	ND	16 ±3	1 ±1
Unvaccinated 3 (Males)(UV3M)	Ocular instillation (0.2 ml; 0.8 mg/ml protein “mock” specimen)	ND	7 ± 2	- 4 ±1

ND - not determined

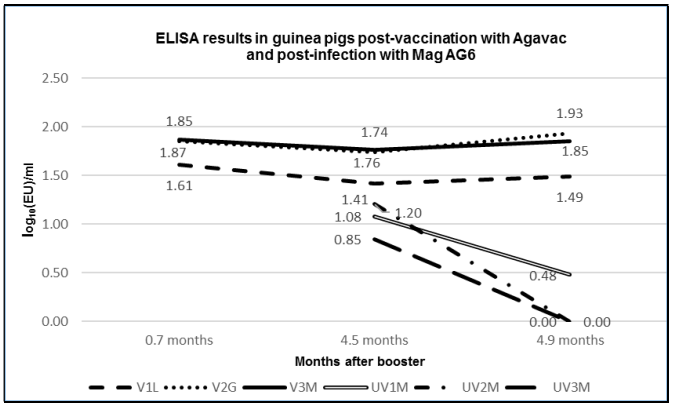

Fig. 1. The dynamics of anti-*Mycoplasma agalactiae* antibody titers in guinea pigs, as evidenced by ELISA, at 0.7 months (21 days) post-vaccination with Agavac, at 4.5 months post booster vaccination (challenge time with Mag AG6 or " mock " test in unvaccinated animals) and 14 days post-infection (4.9 months after booster vaccination). Log10 (30) EU / ml = 1.48 (suspect); log10 (35) EU / ml = 1.54 (positive)

Table 2

The rPCR results regarding the presence of *M. agalactiae* AG6 (Mag AG6) in the organism of vaccinated / unvaccinated animals with Agavac and experimentally infected with Mag AG6, at 14 days post-infection

	rPCR-P40														
Guinea pig group	336 hours (slaughtering)														
	LN	AC	AG	T	R	Sp	F	Cd	P	Cr	Oc	Ov	GM	L	Sg
V1L	-	-	-	ND	-	-	-	-	-	-	-	-	ND	ND	-
V2G	+	-	-	ND	-	-	-	-	-	-	-	-	-	-	-
V3M	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	-
UV1M	-	-	-	-	-	-	-	-	-	-	+	ND	ND	ND	ND
UV2M	-	-	-	-	+	-	-	-	-	-	-	ND	ND	ND	ND
UV3M	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND

Oc - eye swab; R - renal swab; N - nasal swab; L - milk swab; LN - lymph node swab; AC - joint liquid swab; AG – elbow articular liquid swab; T - testicle swab; Sp - Spleen Tampon; F - liver swab; Cd - cord swab; P - Pulmonary swab; Cr - brain swab; GM - mammary gland swab; Sg - blood swab; + positive; - negative; ND - not determined; V1L - Vaccinated guinea pig group 1, Lactating (intra-mammary 4.2 mg Mag AG6 protein / ml; V2G - Vaccinated guinea pig group 2, Pregnant; V3M - Vaccinated guinea pig group 3, Males; UV1M – Unvaccinated guinea pig group 1, Males; UV2M - Unvaccinated guinea pig group 2, Males; UV3M – Unvaccinated guinea pig group 3, Males

Table 3

The rPCR results regarding the presence of *M. agalactiae* AG6 (Mag AG6) in the organism of vaccinated / unvaccinated animals with Agavac and experimentally infected with Mag AG6, at 1, 2, 3, 5 and 9 days post-infection

	rPCR – P40																			
Guinea pigs group	24				48				72				120				216			
	Oc	R	N	L	Oc	R	N	L	Oc	R	N	L	Oc	R	N	L	Oc	R	N	L
V1L	ND	ND	-	+	ND	ND	ND	ND	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND
V2G	ND	ND	+	ND	ND	ND	ND	ND	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
V3M	+	ND	ND	ND	ND	ND	ND	ND	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
UV1M	+	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	-	ND	ND	+	-	-	ND
UV2M	+	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	-	ND	ND	-	-	-	ND
UV3M	-	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	-	-	ND	ND	-	-	-	ND

Oc – eye swab; R – rectal swab; N –nasal swab; L – milk swab; + positive; - negative; ND - not determined; V1L - Vaccinated guinea pig group 1, Lactating (intra-mammary 4.2 mg Mag AG6 protein ND ml; V2G - Vaccinated guinea pig group 2, Pregnant; V3M - Vaccinated guinea pig group 3, Males; UV1M – Unvaccinated guinea pig group 1, Males; UV2M - Unvaccinated guinea pig group 2, Males; UV3M – Unvaccinated guinea pig group 3, Males

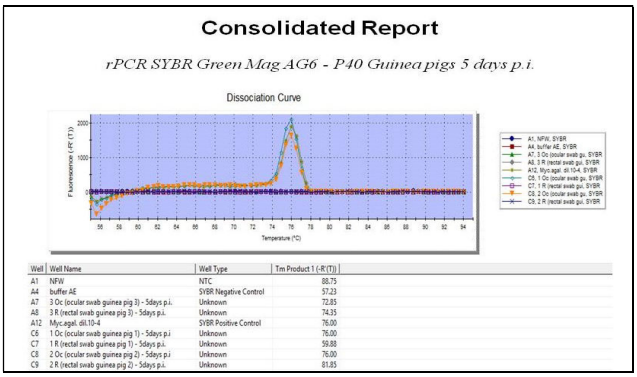

Fig. 2. Detection of *Mycoplasma agalactiae* AG6 by rPCR p40 in experimentally infected guinea pigs, vaccinated / unvaccinated with Agavac, at 5 days p.i. The dissociation curve. Mx3005P spectrophotometric thermal cycler (Agilent - Stratagene). Window from MxPro software

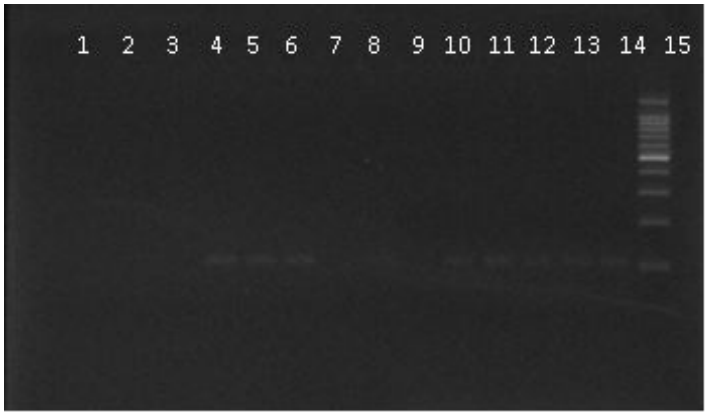

Fig.3. Gel-electrophoresis (TBE1x) for confirmation of rPCR for Mag AG6 p40 detection in guinea pigs swabs, at 2 days p.i. by ocular route (lines 2, 5, 6), 5 days p.i. by ocular (lines 3, 4, 7-10) and 1 day p.i. by mammary/or nasal/ or ocular routes (lines 11-13). Line 1 – negative control, NFW; line 2 – ocular guinea pig 3 (negative, no amplicon); line 3 – ocular guinea pig 3 (negative, no amplicon); line 4 – rectal guinea pig 3 (negative, no amplicon); line 5 – ocular guinea pig 1 (positive, amplicon 109bp); line 6 – ocular guinea pig 2 (positive, amplicon 109bp); line 7 – ocular guinea pig 1 (positive, amplicon 109bp); line 8 – rectal guinea pig 1 (negativ, absenta amplicon); line 9 – ocular guinea pig 2 (pozitiv, amplicon 109bp); line 10 – rectal guinea pig 2 (negative, no amplicon); line 11 – lapte guinea pig 16 (positive, amplicon 109bp); line 12 – nasal guinea pig 19 (positive, amplicon 109bp); line 13 – ocular guinea pig 22 (positive, amplicon 109bp); line 14 – inocul 2 *Mycoplasma agalactiae*

Conclusions

The antibody titers expressed as EU showed statistically significant differences ($p < 0.05$) between vaccinated and unvaccinated groups, at all the tested moments. Mag AG6 colonizes the guinea pig's organism. Mag AG6 inoculated in vaccinated animals at a dose of 5.25 times higher than the dose administered to unvaccinated animals was present at the site of inoculation for maximum 72 hour, as opposed to unvaccinated animals where it persisted until slaughter (13 -14 days pi). The results suggested that the vaccine has given protection (was effective). Agavac vaccine has been shown to be effective (potent / immunogenic and protective) for laboratory animals. Our results proved that the assessment of the efficacy of the Agavac vaccine can also be carried out on laboratory animals (guinea pigs).

Acknowledgements

This project was funded by S.C. Pasteur Filiala Filipesti Srl.

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HISTOLOGY ASPECTS ON KIDNEY AFTER *SILYBUM MARIANUML.* AND *HIPPOPHAE RHAMNOIDES*L. ADMINISTRATION IN RATS WITH ALLOXAN INDUCED DIABETES

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Summary

The growing number of patients with diabetes mellitus has concluded that prophylaxis is the only way to avoid and stop the spread of the disease as well as its complications. Many animal species are affected by this disease, cats and dogs being the species with the highest incidence. In this idea the aim of the research was to highlight the effects of aqueous extracts of *Silybummarianum* and *Hippophaerhamnoides* on the kidney in rats with induced diabetes by intravenous administration of alloxan. Following the histological study of the kidney, it was concluded that the two plant extracts may be recommended as renal protectors and only as a palliative and / or preventive medication for diabetes.

Keywords:kidney, histology, diabetes, alloxan

Diabetes mellitus is a chronic metabolic illness, produced by insufficient secretion of insulin by the beta pancreatic cells or reduced effectiveness of insulin. These pancreas deficiencies can be acquired or inherited. As a result of insulin inefficiency the glucose concentrations in blood increase and eventually lead to damage of the other organs, kidney failure being one of the leading complications (22).

According to the World Health Organization, diabetes mellitus is a disease that affects 150 million people worldwide, and until year 2025 it will double.

Alloxan has the ability to damage pancreatic β-cells responsible for insulin production by inducing formation of free radicals, superoxide radicals the most (7, 14, 23). It is absorbed and accumulated by β-cells because of its structure similar to glucose (1). That is the reason why this substance is commonly used in experiments to induce diabetes in laboratory animals like: rats, rabbits, mice and dogs. Alloxan administration is followed by, regardless of the presence or absence of glucose, sudden increase of insulin secretion. After this short duration release of insulin the response of pancreatic β-cells to glucose lacks completely, even if glucose levels are high (1, 23).

SilybumMarianum L. known as milk thistle is a plant naturally found in Mediterranean basin but it can be cultivated as medicinal plant as well. From ancient times seeds were used in treatment of liver diseases (8). The most valued parts of this plant are seeds because of their high content in silymarin, a mixture of

polyphenolic compounds: silychristin, silydianin, silybin and isosilybin. The studies carried out *invivo* and *in vitro* showed that the value of silymarin is due to its antioxidant, anti-inflammatory, anti-lipid peroxidative, anti-apoptotic, anticarcinogenic, immunomodulatory actions (2, 8). In diabetic patients it reduces hyperinsulinaemia and necessity of exogenous insulin (2).

HippophaeRhamnoides L. known assea buckthorn is a plant commonly found in Europe and Asia. Numerous bioactive substances are found, in high amount, in all plant parts (20). It has been demonstrated that the richest in phenolic compounds are leaves. These phenolic compounds, mainly isorhamnetin and quercetinderivates, are responsible for antioxidant, anti-inflammatory and antitumoral activity (12). Leaves extracts of sea buckthorn also exert immunomodulatory, antibacterial, adaptogenic, radioprotective, and regenerative properties (20). For centuries this plant has been used in traditional medicine in treatment of gastric ulcers, skin disease, asthma, cough and lung disorders (18, 20).

Materialsandmethods

The adult albino Wistar rats used for this study were provided by Animal House of University of Medicine and Pharmacy „Victor Babeş" Timisoara, Romania. The animals were housed in standard cages and laboratory conditions: temperature 25±2°C and illumination 12 h light/dark. The rats had unlimited access to water and were fed with standardized diet.

This study was performed in order to assess the effects of *SilybumMarianum L.* (milk thistle) and *HippophaeRhamnoides L. (s*eabuckthorn*)* aqeous extracts on kidney lesions produced by diabetes mellitus, disease induced by intravenous administration of alloxan.

The materials for aqueous extracts, seeds of milk thistle and fruits of sea buckthorn, were acquired from natural products store. The classic extraction from the plants was performed to obtain the aqueous extracts. Plant particles with size of 0.4 mm, were mixed with distilled water in proportion of 0.6/10 water/volume ratio. The mixture was heated up to 90°C for 10 minutes, and then passed through the filter (16).

According to the experiment design the animals were grouped as follow: control (C) – group which received distilled water and three experimental groups. The dose of 40 mg/kg bw of alloxan (Sigma –Aldich, St. Louis, USA) 2% was administered i.v. in the tail vein to all three experimental groups (3). After seven days the rats with diabetes (glicaemia over 135 mg/dl determined with ACCU-CHEK Active, model GC ROCHE, Mannheim, Germany) were divided in three groups: D - diabetic control, without any treatment receiving only distilled water, SM – group treated with *SilybumMarianum*6% aqueous extract and HR - group which received *HippophaeRhamnoides*6% aqueous extract (13, 16).

After seven weeks of aqueous extracts administration tissue samples from pancreas and kidney were collected for histological examination. For fixation of the

tissue samples 80° ethanol solution was used and then the samples were embedded in the paraffin wax. To obtain histological section 5-micron thickness, the embedded samples were cut using microtome Cut 4062 Slee Mainz, Germany. After staining with Hematoxylin and Eosin method, the histological slides were studied on Olympus microscope CX 41 provided with Olympus digital photo camera and image analysis software QuickPHOTO Micro 2.2.

During acclimatization and experimental periods the animals were housed and cared for in compliance with the laboratory animal welfare national and international legislation.

Resultsanddiscussions

Histological examination of pancreas in control group revealed normal structure of this gland, represented by the exocrine and endocrine parts, organized in lobes and lobules separated by connective tissue rich in sinusoidal and fenestrated capillaries.

Exocrine pancreas is represented by pyramidal (acinar) cells with dark cytoplasm and central nuclei that form serous acinus responsible for secretion of digestive enzymes (Fig. 1).

Endocrine component is made up of Langerhans islets, spherical or ellipsoidal nests with spherical cells - endocrine cells (Fig. 1).

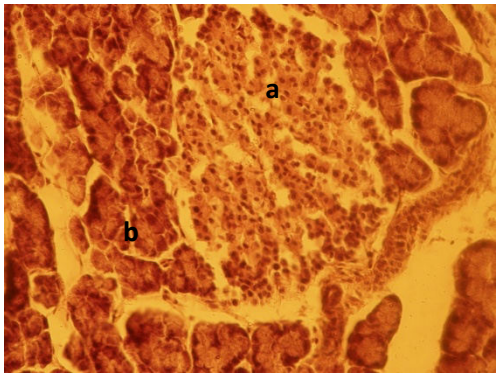

Fig. 1. Histological section of pancreas in control group: Langerhans islet (a) and serous acinus (b), Hematoxylin-Eosin stain, 400X

The microscopic examination of the kidney in the control group highlighted its normal structure, organized in the two areas: the cortex (the outer portion), with a granular appearance due to the presence of the renal corpuscles (Fig. 2) and the medulla (the inner portion), transversally striated due to the presence of the collecting urinary channels.

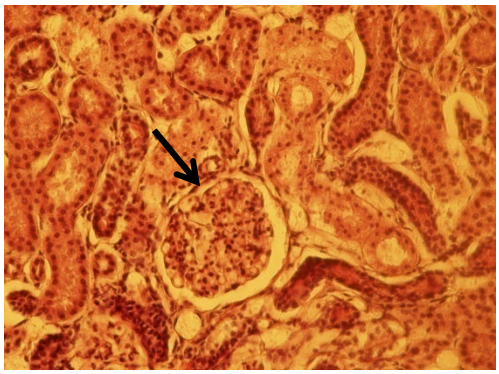

Fig. 2. Histological section of kidney in control group: renal corpuscle, Hematoxylin-Eosin stain, 400X

Histological section of pancreas from rats with alloxan induced diabetes mellitus revealed the installation of the alterative processes characteristic of diabetes, ascertained and presented in our earlier studies, namely: the occurrence of insulitis(Fig. 3), characterized by lymphocytic infiltration in Langerhans islands, of different sizes. In some Langerhans islets there have been edema areas as a consequence of pancreatic beta cell necrosis, which are best represented numerically, thus widening the endocrine cell lines (16). The extension of cytolysis lesions is directly proportional with the edema area.

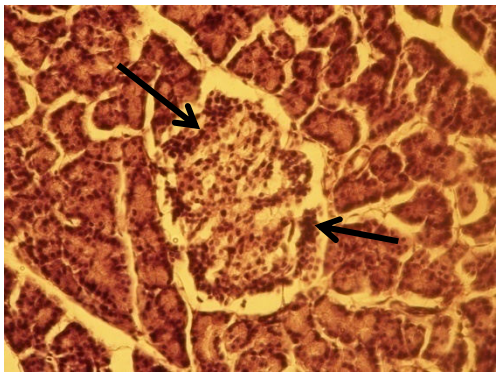

Fig. 3. Histological section of pancreas in alloxan induced diabetes group: insulitis, Hematoxylin-Eosin stain, 400X

The microscopic examination of the kidney in the alloxan-induced diabetes group revealed vascular congestion with large blood vessels and infiltrative and degenerative processes at the nephron level.Vascular congestion is a progressive

complication of diabetes, which will cause hypertension and ischemic nephropathy.

Reducing of the urinary space from the renal corpuscles, probably due to a higher blood flow and/or due to the presence of leukocyte infiltration in the glomerulus and around the urinary tubes, has the effect of dilating them (Fig. 4).

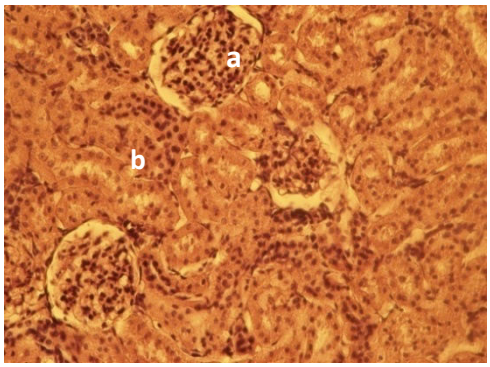

Fig. 4. Histological section of kidney in alloxan induced diabetes group: glomerular (a) and peritubular leukocyte infiltration (b), Hematoxylin-Eosin stain, 200X

Massive leukocyte filtration indicates the installation of interstitial nephritis.

Nephrocytes of the proximal convoluted tubule responsible for the reabsorption of nutrients (glucose, amino acids), water, bicarbonates and electrolytes showed turgescence, with microvilli destruction. Other nephrocytes have been affected by nephrosis, and even by tubular necrosis (Fig. 5).

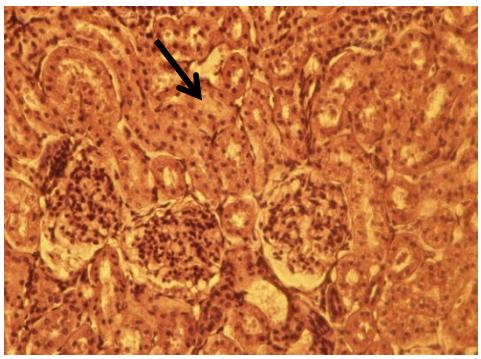

Fig. 5. Histological section of kidney in alloxan induced diabetes group: turgescence of tubules, Hematoxylin-Eosin stain, 200X

The microscopic examination of the kidney in the alloxan-induced diabetes mellitus and treated with the aqueous extract of *Silybummarianum* pointed out

further maintenance of the vascular events: blood vessel dilation and thickening of the arterial walls (Fig. 6).

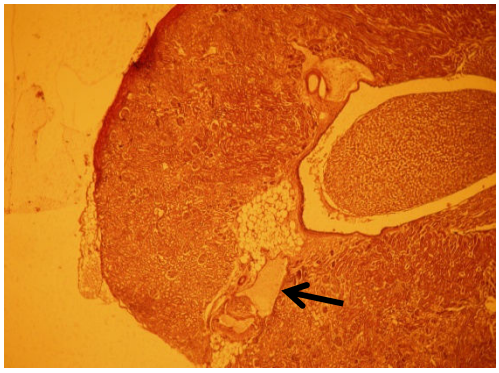

Fig. 6. Histological section of kidney in alloxan induced diabetes group after treatment with aqueous extract of *Silybummarianum*: vascular congestions, Hematoxylin-Eosin stain, 40X

Recovery of blood plasma filtration function was revealed by the withdrawal to the disappearance of leukocyte infiltrate at the level of renal corpuscles, although urinary space was still reduced, as well as the reduction of tubular nephrosis phenomena with the recovery of the absorption function at the level of proximal convoluted tubule (Fig. 7).

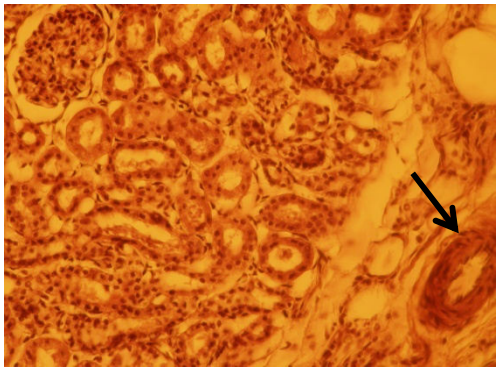

Fig. 7. Histological section of kidney in alloxan induced diabetes group after treatment with aqueous extract of *Silybummarianum*: reduction of leukocyte infiltrate and thickening of the arterial wall, Hematoxylin-Eosin stain, 400X

The microscopic examination of the kidney in the alloxan-induced diabetes group and treated with sea buckthorn aqueous extract (*Hippophaerhamnoides*) revealed, similar to the previous group, the reduction to the disappearance of leukocyte infiltration and of degenerative processes of the nephrocytes in the proximal convoluted tubule (Fig. 9). In this case also, vascular phenomena were present (Fig. 10).

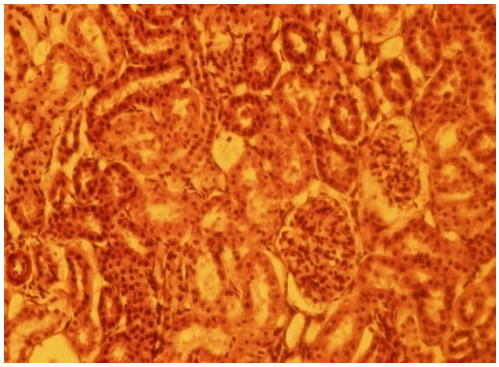

Fig. 9. Histological section of kidney in alloxan induced diabetes group after treatment with aqueous extract of *Hippophaerhamnoides*:normal structural aspect, Hematoxylin-Eosin stain, 400X

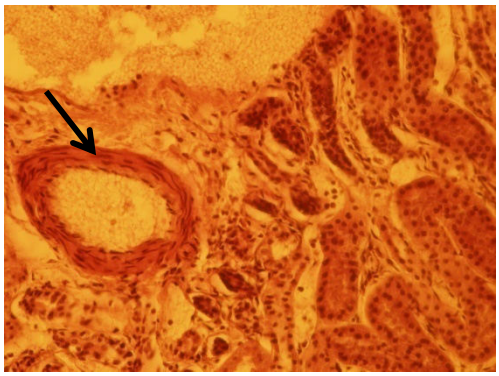

Fig. 10. Histological section of kidney in alloxan induced diabetes group after treatment with aqueous extract of *Hippophaerhamnoides*:thickening and dilation of the blood vessels,Hematoxylin-Eosin stain, 400X

Renal damages described by Soto et al.(2010)after 12 weeks of alloxan administration in rats were glomerular damage with cell disruption and

vacuolization in medulla and cortex, and also tubule lysis. They found that after silymarin administration these lesions, associated with diabetes caused by alloxan administration, were reduced both in cortex and medulla and restored to normal. Also, mesangial matrix increase and capillary basement membrane thickening, the typical lesions in diabetic nephropathy, were blocked.

In alloxan-induced diabetic rats necrosis of beta pancreatic cells is due to the alloxan generation of reactive oxygen species (19, 24). Similarly, Zhu et al.(2005) showed that diabetic nephropathy is due to the reduction of antioxidant enzymes concentrations and activity.

Because of its free radical scavenging activity, silymarin is capable of restoring antioxidant enzymes concentrations and activity after nine weeks of administration (17). Thus renal damages can be restored, renal function improved and diabetic nephropathy prevented (10). Therefore, the aqueous extract of milk thistle can be recommended as a renal protector. For dogs and cats a suggested therapeutic dose of silymarin range between 20-50 mg/kg/day but divided in 3 to 4 doses (21).

The kidney damage caused by alloxan-induced diabetes was reduced almost to disappearance after administration of sea buckthorn aqueous extract. This effect is attributed to antioxidant activity of the composition of sea buckthorn.It was confirmed that sea buckthorn protects against oxidative stress. The antioxidant properties were demonstrated to be effective in liver(4, 6), heart (5), lungs (15), and colon (11). Beneficial effects were also observed after chemotherapy and radiotherapy, when kidney and liver functions were restored (9). Therefore, sea buckthorn extract can also be used as a renal protector.

Conclusions

The lesions produced by the alloxan-induced diabetes in the pancreas are difficult to compensate and require a longer recovery time.

The aqueous extracts of milk thistle and sea buckthorn, respectively, used to treat diabetes-induced lesions by intravenous administration of alloxan may be recommended as renal protectors, as the repair effects observed after histological examination of renal tissue are obvious.

The aqueous extracts from the studied plants can be used in diabetes, only as a palliative and/or preventive medication.

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ELECTROCARDIOGRAPHIC AND ECHOCARDIOGRAPHIC INVESTIGATIONS IN CARDIAC HYPERTROPHY IN CATS, CONSEQUENCE OF HYPERTHYROIDISM

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Summary

Electrocardiographic and echocardiographic investigations were performed in the Functional and Metabolic Exploration Laboratory (CLHC), the Medical Pathology Clinic of the Veterinary Faculty of Timisoara and a private veterinary clinic. Cats of different breeds (European, Persian, Main-Coon), females and males, aged 6.5-7 years and 3.5-5.5 kg body weight, were diagnosed with hyperthyroidism, which can lead to hypertrophic and/or restrictive cardiomyopathy.Electrocardiographies were performed with the Contec Electrocardiograph, model 300 GA, with 12 derivations, thermal printer and 80 mm ECG paper, and the parameters used were 25mm/sec and 10mm = 1mv.The echographic investigations in B, M, Color Doppler and Spectral Doppler mode were performed with a MyLab 70 VET XVG stationary ultrasound and two portable unltrasounds, one Vivid I General Electric, and the other, the Mindraz 2200 VET.Electrocardiography revealed aspects of sinusal tachycardia, hypertrophic cardiomegaly by excessive amplitude increase of the atrioventricular complexes, with the possibility of adaptive disorders, considering the increased frequency and terminal phase changes, the T wave signifies repolarization disorders (increase of the passive filling period). Also, the presence of an increased amplitude wave, with supraventricular origin (nodal extra systole), without pathological connotation, was found in all derivations.Two-dimensional cardiac ultrasound (Module B), in the right parasternal section, with two chambers, in transversal section revealed a left atrium/aortic ratio of 0.92, which physiologically should be higher, the interventricular septum in diastole (IVSED) measuring 0,45 cm and in systole (IVSES) 0.82 cm, left ventricular wall free (LVW), hypertrophic interventricular septum (IVS), aortic stenosis aspect.In the M mode, there is an increased compliance between the interventricular septum and the left ventricular wall, which leads to incipient phase cardiac hypertrophy, reducing the left ventricular cavity.By color Doppler ultrasound, a turbulent transmitral flow was observed, and the spectral module revealed a normal left ventricular diastolic image, a slightly restrictive isovolumetric relaxation time, and the ratio between rapid ventricular filling and atrial systole was increased.Blood biochemistry revealed high T4 values ranging from 4.2-5.2 ng/dl, and low TSH values, between 0.012 - 0.032, in all cases.

Keywords: echography, electrocardiography, cat, cardiac hypertrophy, hyperthyroidism

The cases' number of cardiac disorders in cats, in general, and in particular myocardial disorder cases are increasing, either due to repeated inbreeding, especially in improved breeds, or due to uncontrolled pairing. Generally, the number of individuals in the feline population with clinical manifestations of the cardiovascular system is relatively low. However, incipient cardiomyopathy stages may be a surprise following clinical and paraclinical (haematological, biochemical, echocardiographic, electrocardiographic) examinations.

Physiologically, in cats, due to the morphological aspect of the chest, the heart has a high and elongated silhouette and, due to thyroid hypersecretion, the risk of hypertrophic cardiomyopathy is increased (4, 6).

In support of the early diagnosis of this myocardiopathy and its staging, M-mode and B-mode echocardiography is useful in diagnosing ventricular hypertrophy in cats and differentiating myocardial structure values rom the upper physiological limit of as compared to pathological values. On the other hand, echocardiographic examination in the Color Doppler and spectral mode provides useful information about the presence of turbulences and the velocity of blood flow at heart level (1, 2, 3)

The ECG in cats as a non-invasive method is needed to identify hypertrophic cardiomyopathy, cardiac pace and cardiac conduction disorders (5).

Considering the above mentioned, we have proposed ultrasound and electrocardiographic investigation in cats with cardiac hypertrophy as a consequence of hyperthyroidism.

Materials and methods

Electrocardiographic and echocardiographic investigations were performed in the Functional and Metabolic Exploration Laboratory (CLHC), the Medical Pathology Clinic of the Veterinary Faculty of Timisoara and a private veterinary clinic.

Cats of different breeds (European, Persian, Main-Coon), females and males, aged 6.5-9 years and 4-6.3 kg body weight, diagnosed with hyperthyroidism, which can lead to hypertrophic and/or restrictive cardiomyopathy.

Electrocardiographies were performed with the Contec Electrocardiograph, model 300 GA, with 12 derivations, thermal printer and 80 mm ECG paper, and the parameters used were 25mm/sec and 10mm = 1mv.

The echographic investigations in B, M, Color Doppler and Spectral Doppler mode were performed with a MyLab 70 VET XVG stationary ultrasound and two portable ultrasounds, one Vivid I General Electric, and the other, the Mindraz 2200 VET, on vigilant animals in standing position or lateral decubitus. A microliniar probes with a frequency of 6.5 MHz and amicro convex probe with frequency of 5MHz were used.

Results and discussions

The electrocardiographic and echocardiographic aspects of a 6.5 year old female Persian cat, weighing 5.5 kg, clinically presenting dyspeptic episodes, inappetence, weakness, mild oral dyspnea, an increased cardiac area, intensified heart sounds and increased heart rate, and, according to blood biochemistry, elevated thyroid hormone levels -T4 (4.3 ng/dl) and low thyroid hormone-TSH (0.025 ng/ml) are shown in Fig. 1, 2, 3.

Following the analysis of the electrocardiographic route, there were aspects of sinus tachycardia, possibly by accentuating metabolism secondary to the increased thyroid hormone levels, which leads to the increase of the cardiac mechanical work, favoring the adaptive phenomena, especially the hypertrophic ones, with cardiac insufficiency in the end. Considering the relatively normal aspects of the ventricular complex (QRS), we can say that no visible electrocardiographic changes were observed (Fig. 1).

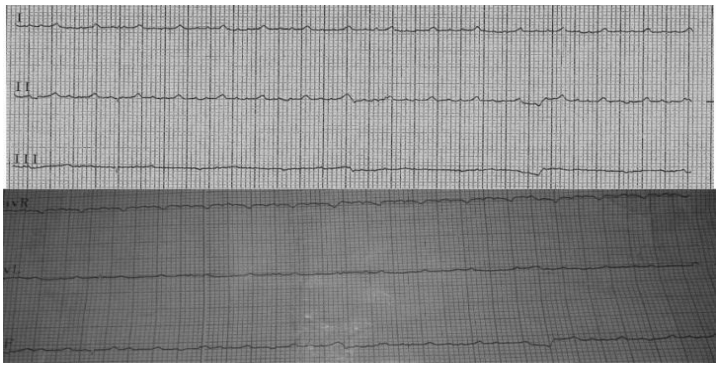

Fig. 1. Electrocardiogram in cats, in uni- bipolar derivatives

Two-dimensional cardiac echocardiography (mode B) in right parasternal section, with two chambers, transversal section revealed a left atrium/aorta ratio of 0.92, which physiologically should be higher. This signifies mild hypertension in the cardiac section of the aorta. Also, the interventricular septum during the diastole (IVSED) recorded 0.45 cm and 0.82 during the systole (IVSES).

After Brăslaşu (2008), the value of the interventricular septum during the cardiac revolution is above the physiological limit (0.31/0.58 cm), which represents hypertrophy of the interventricular septum. Concerning the posterior left ventricle wall during the systole and the diastole, named PVSS/PVSD, they had values of 0.73 cm and 0.49 cm.Following thedetermination of diastolic (DD) and systolic (SD) diameter values, the shortening fraction (SF) was calculated to be 44%, which means a physiological contractile and adaptive capacity (Fig. 2, 3).

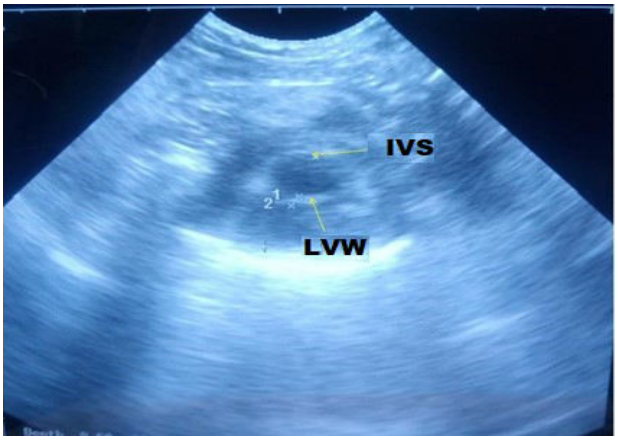

Fig. 2. B-mode ultrasound in cats, right parasternal window (transversal section), with two chambers

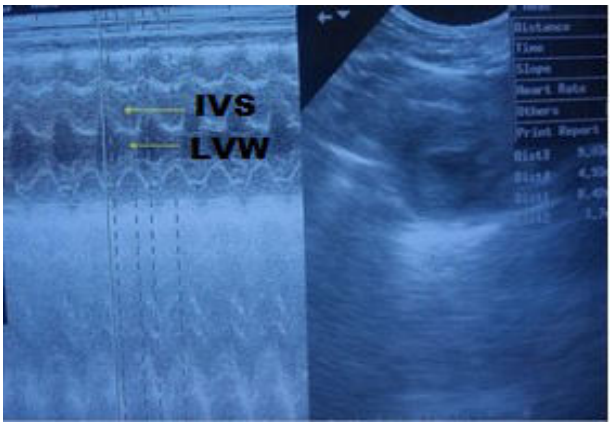

Fig. 3. M-mode ultrasound in cats, left parasternal window (transversal section), with two chambers

In a 7-year-old European male feline that experienced fatigue during effort, decubitus, dyspnea, oral breathing, cooling of the extremities with cyanosis of the paw skin, lack of pulse, increased heart rate and cardiac sounds, with T4 values of 5.2 ng/dl and TSH values of 0.012 ng/ml, electrocardiographic investigations performed in right lateral decubitus reveal aspects of sinus tachycardia and

hypertrophic cardiomegaly due to excessive increase of the atrioventricular complex amplitude (Fig. 4).

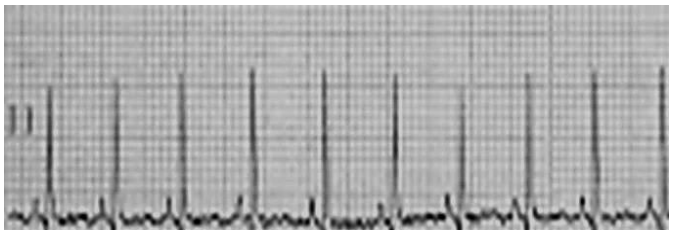

Fig. 4. Atrial tachycardia with high R waves in a cat with cardiac hypertrophy

Two-dimensional B-mode cardiac ultrasound revealed a free left ventricular wall (LVW), a hypertrophied septum interventricular (IVS), and a subaortic stenosis aspect (Fig. 5).

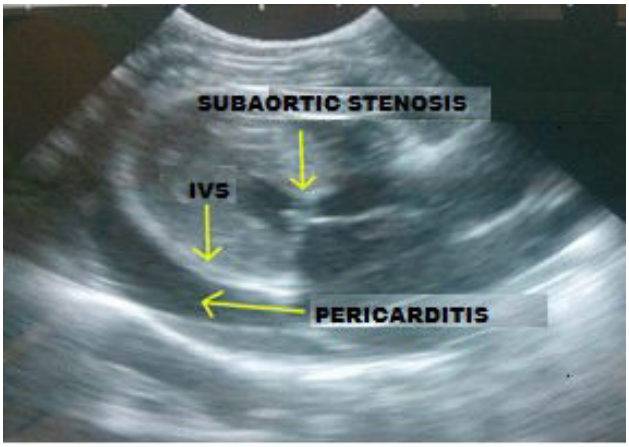

Fig. 5.Cat Echocardiography (B-mode), right parasternal window, longitudinal section, with three chambers

As for the left atrium, it is found dilated, which is suggestively called "atrium attached to the ventricle" (Fig. 6).

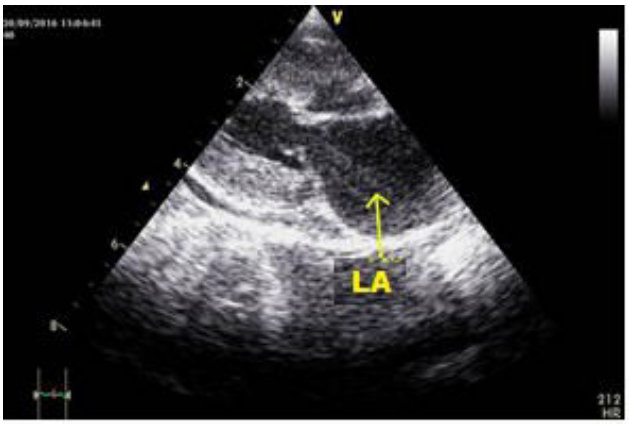

Fig. 6.Cat Echocardiography (B-mode), right parasternal window, longitudinal section, with three chambers

The structures examined previously were dynamically highlighted by M-mode echocardiography, observing that the mitral valve does not exhibit morphological or functional changes. However, the decrease of the ventricular cavity, due to concentric hypertrophy at the level of the papillary muscles, is visible (Fig. 7). Other researchers note that the interventricular septum has a value of 1.16 cm, compared to the maximum admissible value of 0.76 +/- 0.12 cm (2).

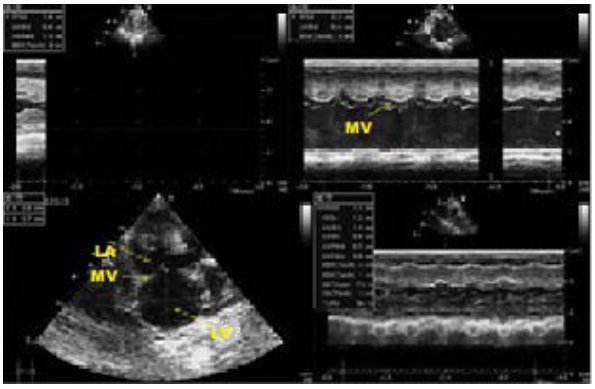

Fig. 7. Cat echocardiography (M-mode), right parasternal window, at the levels of the ventricle, left atrium and mitral valve

The examination of the mitral valve by the Doppler technique reveals a turbulent blood flow, but in conjunction with its appearance in the M-mode, where no changes were noted, we can state that the current regurgitation is due to the architectural modification of the mitral ring, secondary to papillary hypertrophy, together with a descendant laminar flow from the ventricle to the atrium (Fig. 8).

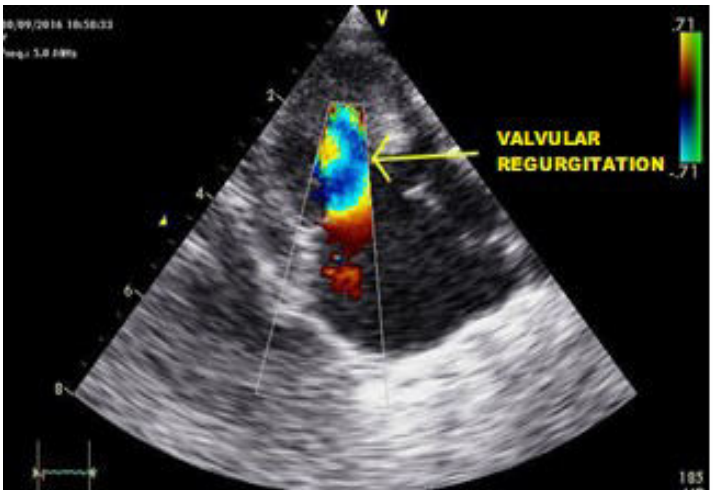

Fig. 8. Cat Echocardiography, color Doppler mode - valvular regurgitation

In the Main Coon 6 year old cat, good conformation, robust structure, lymphatic temper with mild symptoms (cyanosis periods, secondary to increased effort intensity, dyspeptic syndrome with vomiting episodes, slightly increased heart sounds, organic endocardial stenosis sounds), electrocardiogram performed in right lateral decubitus does not reveal characteristic changes of cardiac hypertrophy, but cannot be excluded due to the existence of the adaptive myocardial model which appears in excentric cardiac hypertrophy (6)

However, the ventricular complex QRS is widened, the ventricular complex is negated in D2, D3, Avf, its position in Avr and Avl, the absence of T waves in all derivatives, the arrhythmia of the ventricular complexes, characteristic aspects for the right branch block (RBB) and atrial fibrillation (Af) (Fig. 9). In the D3 derivative, the ventricular complex is superior to the species values, being specific for left ventricular enlargement.

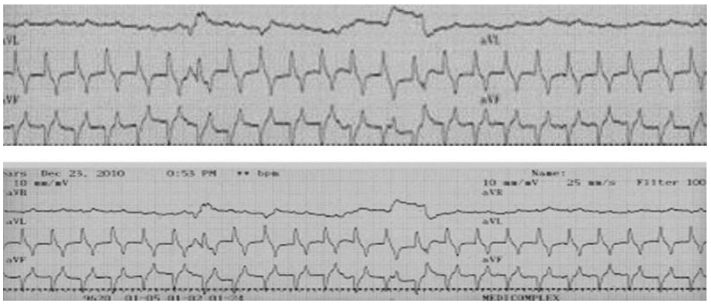

Fig. 9. Atrial fibrillation associated with right branch block in cats

The echocardiography performed in both right lateral decubitus and standing position using the right parasternal window, long axis, B-mode, reveals the presence of the integral pericardial sac, without collection, free ventricular wall and slightly hypertrophic interventricular septum with an emphasis in the upper third, which presents endocardial hypercogenic regions, suggesting mildsubendocardial stenosis (Fig. 10).

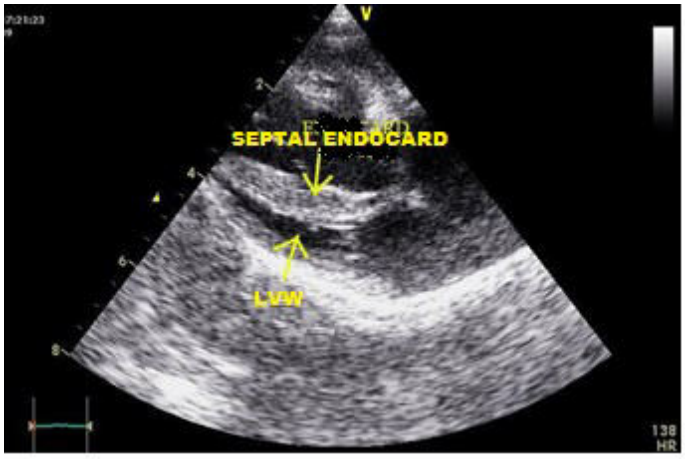

Fig. 10.Echocardiography of a cat (B-mode) - right parasternal section

M-mode echocardiography highlights an increased compliance between the interventricular septum and the free left ventricular wall, features characteristic of cardiac hypertrophy in the incipient phase (Fig. 11, 12).

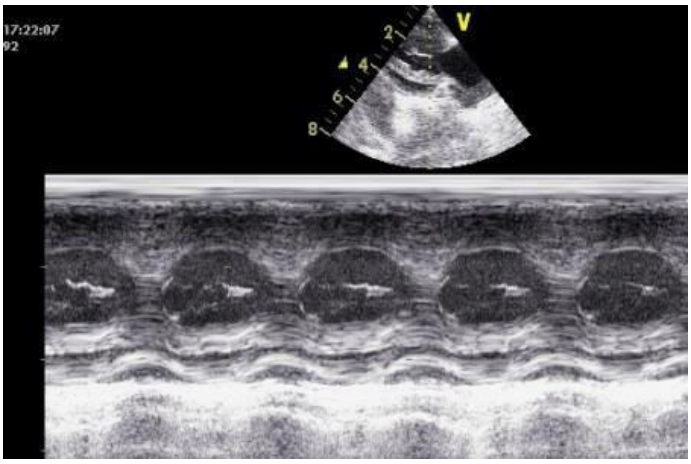

Fig. 11.Echocardiography (M-mode) in cats, at the levels of the interventricular septum, left ventricular cavity and free left ventricle wall

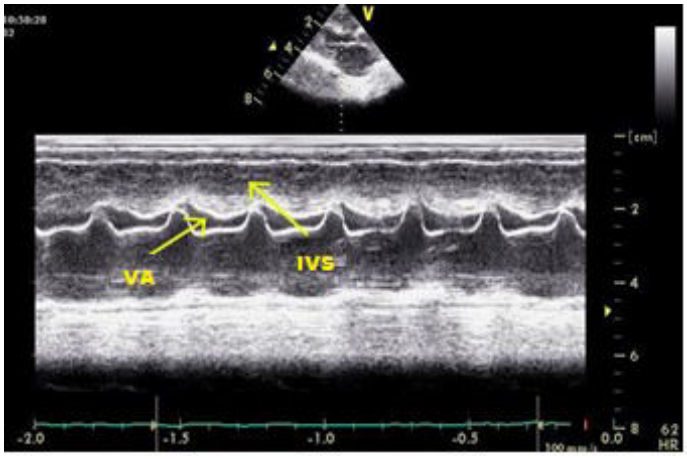

Fig. 12. Echocardiography (M-mode) in cats, at the levels of the interventricular septum and the aortic valve

Spectral Doppler ultrasound examination technique revealed a normal left ventricular diastolic image, slightly restrainedisovolumetric relaxation time, and the ratio between fast ventricular filling and atrial systole was increased. These aspects show slightly restrictive cardiopathy, decreased ventricular compliance, with direct effect on hemodynamics (Fig. 13).

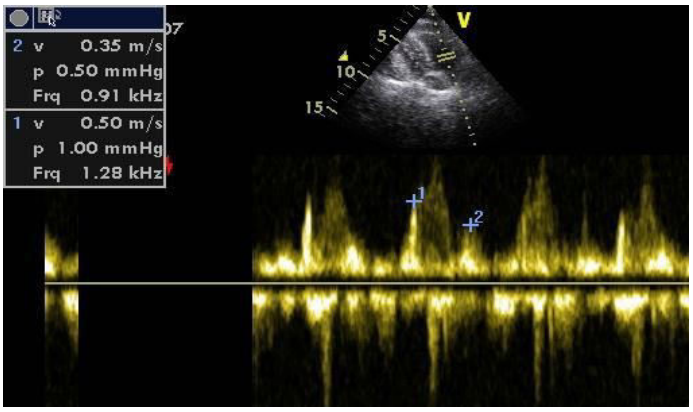

Fig. 13. Cat ultrasound, spectral Doppler specimen at the level of the left ventricle

Blood biochemistry showed increased T4 values (4.2 ng/dl) and low TSH values (0.02 ng/mL).

ECG in the 7-year-old European male feline, which clinically showed fatigue during medium intensity effort, weight loss, polypnea periods, arrhythmias, high intensity and high frequency cardiac shock, with T4 values of 3.8 ng/dl and TSH values of 0.032 ng/mL, revealed aspects of incipient cardiac hypertrophy, with the possibility of adaptive disorders in terms of increased frequency, terminal phase changes of the T wave. These aspects signify repolarization disorders, more precisely increasing the period of passive filling. Also, in all derivatives there is a presence of an increased amplitude wave, with supraventricular origin, called nodal extra systole, but no pathological meaning in this specific case (Fig. 14).

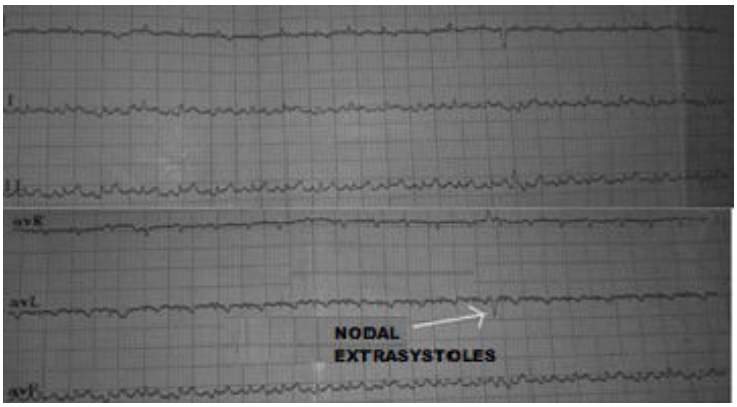

Fig. 14. Electrocardiogram in cats - sinus tachycardia and nodal extra systoles

By M-mode echocardiography, using the right parasternal window in the long axis, 4-chamber section, the following structures were visualized: hyperecogenic integral pericardium, free left ventricular wall (LVW), left ventricular cavity (LV) and interventricular septum without changes, and the cavity of the right ventricle and its wall also without pathological changes (Fig. 15).

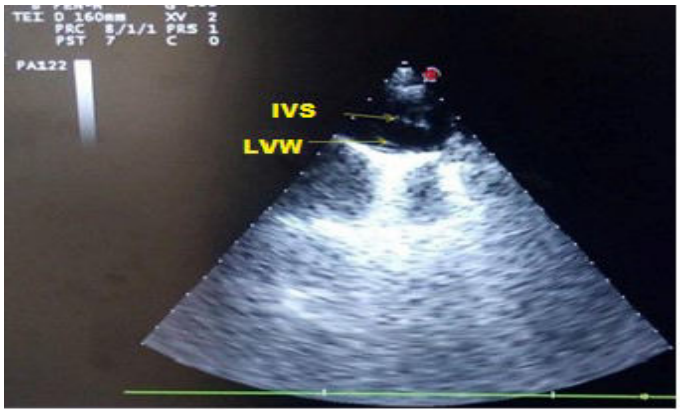

Fig. 15. B-mode echocardiography, right parasternal section, long axis - in cats

The M-mode examination was performed by fixing the volume sample at the level of the interventricular septum and the left ventricular cavity, observing the reduction of the left ventricle cavity and of the compliance between the left ventricle wall and the interventricular septum (Fig. 16).

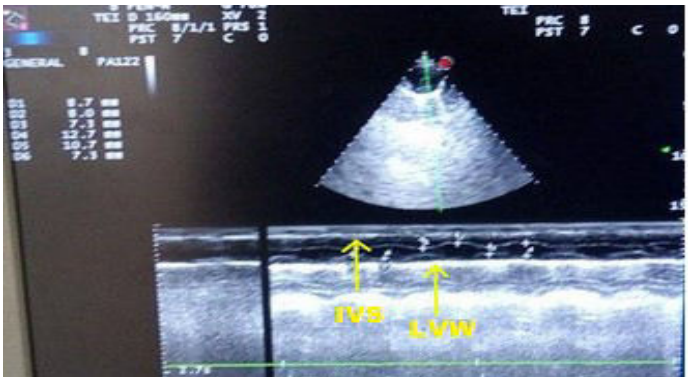

Fig. 16. M-mode echocardiography -the interventricular septum and left ventricle wall in cats

In the color Doppler examination mode, by fixing the sample to the aortic ejection tract and the left ventricle cavity, no circulatory turbulence were identified, showing a clear blue hue (Fig. 17).

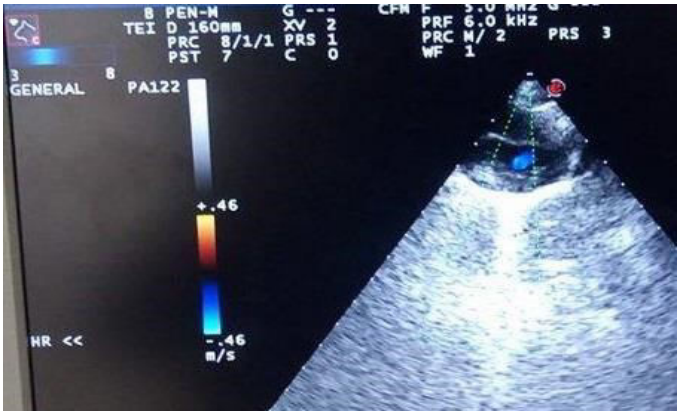

Fig. 17. Color Doppler ultrasound showing the left ventricle and the aorta in cats

Spectral Doppler examination in order to evaluate the diastolic function of the left ventricle revealed a normal isovolumetric relaxation period (TRIV) and the rapid filling and atrial systole represented by the E-wave, respectively the A-wave. Also, the E/A ratio falls into the physiological aspect, with the specification that the slope of E wave is "steep". In fact, it is a slightly restrictive aspect of the left ventricle (Fig. 18).

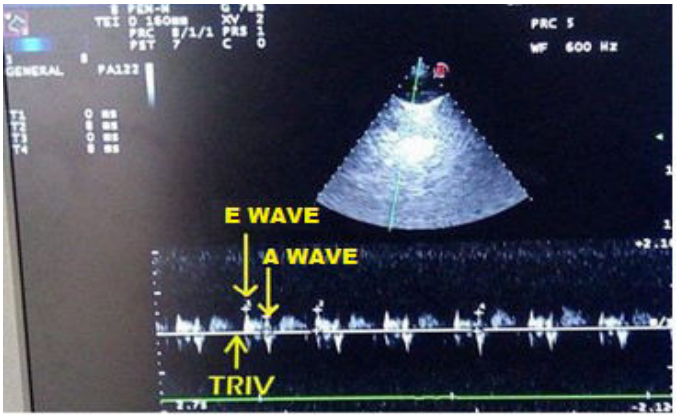

Fig. 18.Spectral Doppler showing the left ventricle in cats

Conclusions

Blood biochemistry showed increased T4 values (4.2-5.2 ng/dl) and low TSH levels (0.012-0.032) in all cases under study.

Electrocardiography revealed aspects of sinusal tachycardia, hypertrophic cardiomegaly by excessive amplitude increase of the atrioventricular complexes, with the possibility of adaptive disorders, considering the increased frequency, the terminal phase changes and the T wave.

These aspects mean repolarization disorders, namely the increase of the passive filling time.

All derivatives showed the presence of a high amplitude wave with supraventricular origin (nodal extra systole), without pathological meaning.

Two-dimensional cardiac ultrasound (B-mode), in the right parasternal section, with two chambers, transversal section revealed a left atrium/aorta ratio of 0.92, which physiologically should be higher, interventricular septum during the diastole (IVSED) of 0.45 cm and during the systole (IVSES) of 0.82 cm., free left ventricular free (LVW), hypertrophied septum interventricular (IVS), aortic stenosis aspect.

In the M-mode, there is an increased compliance between the interventricular septum and the free left ventricular wall, which leads to early stage cardiac hypertrophy, reducing the surface of the left ventricular cavity.

By color Doppler ultrasound a turbulent transmitral flow was observed, and using the spectral mode showed a normal diastolic image of the left ventricle, a slightly restrictive isovolumetric relaxation period, and the ratio between fast ventricular filling and atrial systole was increased.

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A THREE YEAR RETROSPECTIVE STUDY OF CANINE PARVOVIROSIS IN CLUJ COUNTY

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Summary

Canine parvoviral disease is regarded worldwide as one of the most important infectious diseases encountered in dogs due to the severity of clinical signs and the lethal course. This study was aimed to investigate the epidemiological characteristics of CPV infection affecting dogs from Cluj County during a 3 years period (from December 2016 to February 2019). A total number of 144 clinical cases were included in this study, both females and males, from different breeds and ages. The protocol included clinical and hematological evaluation of the suspect animal, while the confirmatory diagnosis was based on the positive result of commercial snap tests. Data collected using questionnaires (history, age, breed, possible exposure, vaccination status) and medical forms filled for each case were statistically analyzed. The retrospective analysis indicated an increasing number of cases during the studied period of time and severe course of the disease for the great majority of the cases. Compared to literature, our results indicated that both the non-vaccinated and vaccinated dogs were at risk to develop the disease. Similar to other studies, the age was determined as a risk factor. The study underlines the importance of both vaccination and disinfection in order to reduce the animals exposure and environment contamination.

Keywords: parvovirosis, dogs, epidemiology, risk factors

Canine parvovirosis is the most frequent infectious disease encountered in young dogs. The disease is produced by a highly contagious virus that causes increased mortality, with a fast-clinical course (7). The virus is small non-enveloped, containing a single stranded DNA of circa 5.2 kb in length (8). The capsids of the virus are icosahedral and one capsid contains 60 protein subunits of VP1 and VP2, all sharing the same structure, being produced by the alternative splicing of the viral mRNAs (15). For cellular uptake the parvovirus uses endocytosis, as well as glycoproteins, glycans and glycolipids. Therefore the choice of the receptors will determine host specifity and tissue tropism (5). The capsid has a central core that is composed of an eight-stranded, antiparallel β-barrel that has flexible loops between the β-strands. These before mentioned β-strands are the ones forming the surface of the capsid. The surface has a 22 Å spike on the threefold axes as well as a 15 Å deep canyon that surrounds the cylindrical structures at the fivefold axes. In addition to this it has a 15 Å deep “dimple” at the twofold axes. Interesting enough the most antigenic region of the capsid are the threefold axes, which serve as the antibodies neutralizing targets (8).

The literature findings have shown that the disease is encountered only in puppies of 6 to 12 weeks old, because the slightest older dogs are protected via maternally induced immunity (2). A study conducted by Prittie in 2004 has shown that the more susceptible dogs are the ones with ages between 6 weeks and 6 months of age are the ages that they are the most unprotected for the action of the virus. In regards to the breed specificity, studies have shown that certain breeds present a higher sensitivity to it. Amongst the breeds Rottweilers, Pitbull terriers and German Shephard are cited as being the most at risk (10). Contrary to that research, other authors have declared that Pinchers and Labradors Retrievers are breeds that have a higher sensibility to the action of the virus (11). Goddard and Leisewitz (4) proved that not neutered male dogs have a twice as big of a risk of contracting the disease than sexually intact females (4)

The contamination can occur through two ways, a direct one and an indirect one. The direct one takes place through fecal-oral route and the other one is via exposure to infected objects, clothes, people. A very important factor to take into consideration is that the fecal excretion of the parvovirus will take place for up to 4 weeks after the clinical signs have been ameliorated.

The replication of the virus takes place in the lymphoid tissue of the mesenteric lymph nodes, thymus and oropharynx. Via hematogenous spread the parvovirus spreads into the intestinal crypts of the small intestines. (6).

Diagnosis of this pathology is usually based on clinical signs, although literature cites the use of commercial snap tests (3). This test is a rapid enzyme immunoassay that detects the canine parvovirus antigen in the canine feces. The company that manufactures the tests have a sensitivity and a specificity of 95 % (Idexx laboratories).

In terms of the treatment of this disease, it being a virosis, the treatment is strictly symptomatic. Fluid therapy is highly important in order to combat dehydration and electrolyte imbalance. It usually consists in a mix of a sterile, balanced electrolyte solution, with of B-complex vitamins, crystalloid or colloid fluids. In addition to this antiemetics and broad-spectrum antibiotics are also used. Some authors recommend enrofloxacin as a broad spectrum antibiotic but most of the clinicians don't use it because it affects the growing cartilage (7).

Luckily, parvovirosis can be preventable through vaccination and rigorous disinfection (10, 12).

Materials and methods

A total of 144 dogs were taken into study during a time period of 3 years, from December 2016 to February 2019. The dogs were both male and female, of different ages starting at one and half months leading up to twelve months old. Regarding the breeds of the dogs, the patients were composed of a high variety of breeds.

Our protocol included clinical and hematological evaluation of the suspect animal, while the confirmatory diagnosis was based on the positive result of commercial snap tests. The information collected using questionnaires that comprised of the age, breed, vaccination status and history of the patients were used to help us analyze the data. The retrospective analysis indicated an

increasing number of cases during the studied period of time and severe course of the disease for the great majority of the cases.

Results and discussions

From the total of cases, a percentage of 51 % were positive to the disease, testing done by using the commercial snap tests (IDEXX, USA). The rest of the cases, 49 % were negative (Fig.1).

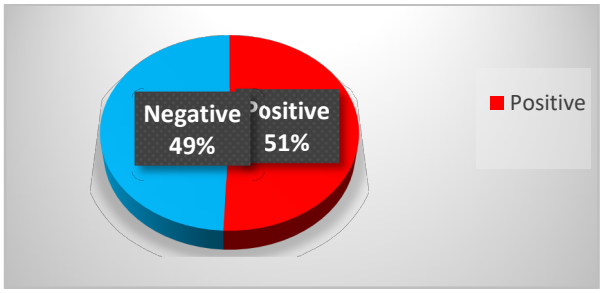

Fig. 1. Total of snap test diagnosed cases

Regarding the age of the dogs, the dogs included in the study had ages between 1,5 months old and 12 months old. As far as that goes, the most frequent age of the diagnosed dogs in our clinic was 6 months old (no= 17), followed by 4 and 3 months old (no=14;13). The least number of diagnosed cases was at the age of 9 months (no=1) (Fig. 2)

In figure 3 we can observe the prevalence of the cases regarding the gender of the patients. The literature says that more males are diagnosed with parvovirosis, as opposed to the female patients. Our study comes to support the literature findings, with 58% of the cases being comprised of male patients and 42% of female dogs.

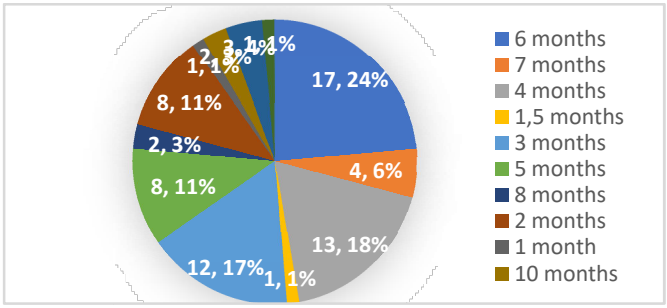

Fig. 2. Positive dogs by age

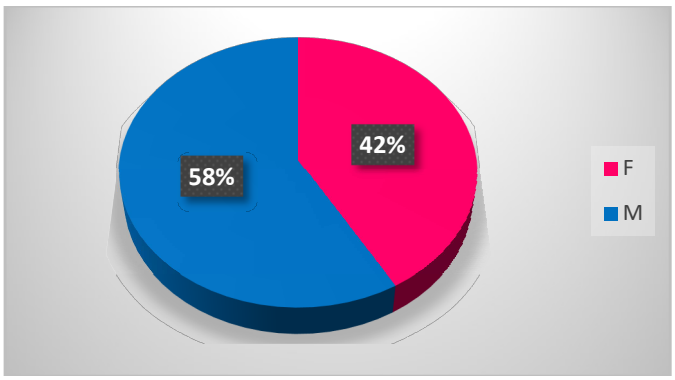

Fig. 3. Gender of diagnosed patients

In the past parvovirosis was normally diagnosed only to non vaccinated dogs, but lately more and more cases of this disease appear to vaccinated dogs. Our study is consitant with the research, meaning that 51% of the dogs diagnosed were not previously vaccinated against parvoviros, while the rest of the 49% were vaccinated (Fig.4).

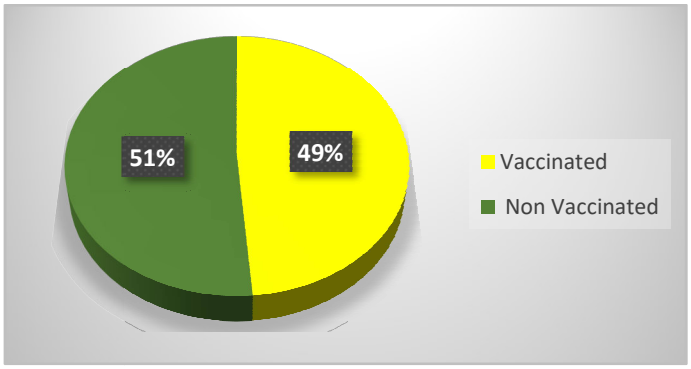

Fig. 4. Vaccination status of diagnosed patients

The suspected cases that came into our clinic were from 27 different cities from Transilvania. The highes ponder of cases came from our city, Cluj-Napoca (No=90), followed by Apahida, 13 cases. From this total of cases, 41 one cases from Cluj-Napoca were diagnosed and the rest of them were coming from different cities in the selected area (Fig. 5) (Table 1).

Research has shown that the most frequently affected breeds are German Shepherd, Labrador and Golden Retriever. Our study comes to argue with that, meaning that most cases diagnosed with parvovirosis were to mixted breed dogs. This may be because they are the most frequently encountered dogs in our country. In figure 6 we can observe that a high percentage of the cases are represented by pure breed dogs and in table 2 we can obseve that the german

shepherd takes the second place as the frequency, but Golden Retriver, although a popular breed in our region cannot be taken into consideration.

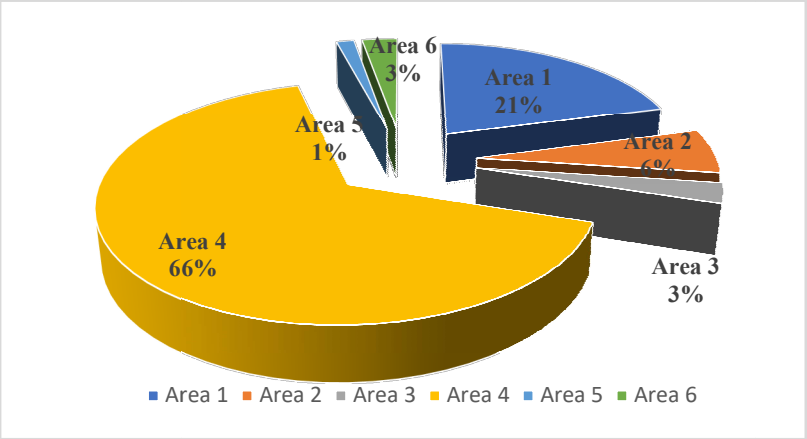

Fig. 5. Location of the diagnosed cases

Table 1

Areas of provenance of diagnosted cases

Area 1	Area 2	Area 3	Area 4	Area 5	Area 6
Apahida	Aghireș	Blaj	Cluj-Napoca	Cămărașu	Baciu
Cojocna	Bonțida	Mediaș	Feleacu	Urca	Florești
Căianu	Deușu	Sibiu	Turda		
Dej	Gilău				
Dezmir	Hjuedin				
Gherla	Mănăstireni				
Jucu	Vlădeasa				
Sânicoară	Zalău				
	Arad				

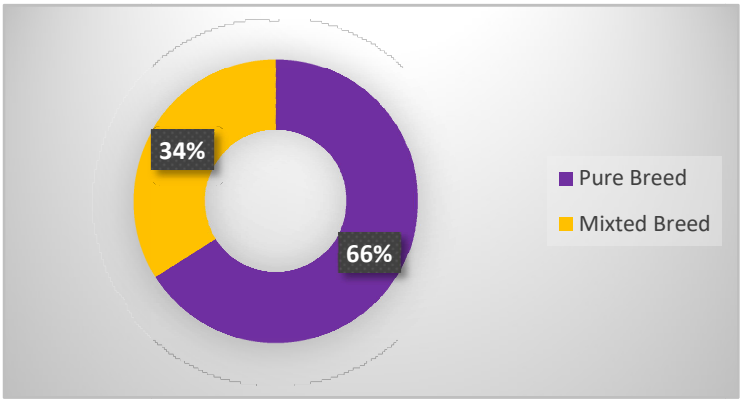

Fig 6. Percentage of diagnosed cases based on breed

Table 2

Breed of the diagnosed cases

Breed	Number of cases
Mixed	26
Amstaff	1
Cocker Spaniel	1
Bichon	10
German Shepherd	7
Central Asia Shepherd	4
Labrador	4
American Buldog	1
Beagle	1
Rottwailler	3
Teckel	2
Romanian Shepherd	1
Pekignez	2
Epagneul Breton	1
French Buldog	1
Yorkshire Terrier	4
Pointer	1
Jack Russell	2
Lagoto Romangollo	1
Belgian Malinois	2
Hound	1
Bucovina Shepherd	1

Conclusions

A large number of cases are suspected of canine parvovirosis lately. What is interesting is that contrary with the past not even the vaccinated cases can contract the disease, being later diagnosed with it. The high percentage of vaccinated cases that were positive to parvovirosis, namely 49%, can raise questions regarding either the vaccination protocols, the vaccine itself, or even the strains of virus involved in the onset of the pathology.

Similar to the literature, it appears that the gender of the patient can influence the onset of the disease. It seems like male dogs are more prone to developing the disease than the female dogs.

Our study shows that dogs up to the age of 12 months can get sick with the parvovirus, with a peak at the age of 6 months. As far as the breed goes it really depends on which breeds are most commonly found in the regions taken into the study. Therefore our study is contradictory to the research previously done.

In conclusion, the high number of diagnosed cases indicated that parvovirosis remains one of the often-infectious diseases that appear in young dogs, the only thing that changed is that now the positive diagnosis can be also put to vaccinated dogs.

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COMPONENT SPECIES IDENTIFICATION FROM DIFFERENT DAIRY PRODUCTS

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Summary

In the last decades, DNA techniques started to be used for component species identification in food and feed of animal origin. These techniques present the advantage of the high sensitivity and also specificity of (PCR)-based on methods to detect very low amounts of DNA from undeclared material that may be fraudulent added in even dairy products. More often the adulteration of dairy product is made by adding cow milk in products prepared from the milk of other ruminant species. This paper work describes a study of detecting the component species from different milk products. Samples were collected from the local market and the composition was unknown. DNA was extracted and purified starting from low amount of sample and it was subjected to PCR analysis with primers specific to cow, buffalo, goat and sheep.

Keywords: ruminants, species identification, PCR based method, dairy products

Most of the dairy products that are present in autochthon market are made from cow's milk. Goat and sheep cheeses are considered to be specialties that have special sensory qualities, noting the taste and aroma. In the production of these particular products, sometimes an attempted to replace the raw material represented by the goat or sheep milk with cow's milk can be noticed without to be declared in the product label (1, 7).

Sheep and goat milk production is of particular economic importance as a result of the search on the market for these traditional cheeses. The fraudulent addition of undeclared milk during the technology process has proven to be a common practice that can cause problems for reasons such as intolerance or allergies, ethical or cultural objections, and legal requirements (7).

For the detection of adulteration various methods have been developed such as immunology, electrophoresis, chromatography and also DNA based methods. The European Community currently has the reference method for the detection of cows' milk and caseinates in cheese produced from sheep's milk, goat milk, buffalo milk or mixtures thereof is isoelectric focusing of gamma-casein after plasminolysis (5).Additionally, several new methods, either immunological (3) or electrophoretical techniques (3,4), have been published recently, which might be applicable to routine analysis.

However, protein-based methods for species identification may fail after excessive proteolysis or heat-induced denaturation of the indicator proteins.

Genomic DNA from somatic milk cells is suggested to persist in ripened cheese and may be amplified and analyzed for species discrimination. Therefore, in order to avoid possible fraud by replacing other species milk and with cheaper cow's milk, it is necessary to develop analytical methods capable of detecting fraud and protecting consumers against counterfeit labels (4), against the fraudulent practices observed more often in the food industry (2, 6).

The purpose of this article is to highlight the importance of authentication of dairy products, in particular the application of the modern and accurate PCR analytical method to the detection of adulteration by exposing a method of detection for the addition of cows' milk to dairy products labeled as other ruminant species milk.

Materials and Methods

The biological material consisted of a total of 10 dairy products (milk, cheese, yogurt, sour cream) existing on the domestic market were purchased, varying in composition, recipe, producer and provenance. In their choice was followed that the label had mentioned the milk of the three types of species and that they are covering a large range of dairy products (Table 1).

Table 1

Biological samples used in this study

Nr. Crt	Biological sample	Provenience/type
1.	Milk	Traditional product
2.	Sour cream 12% fat	Hypermarket/commercial product
3.	Sour cream 12% fat	Hypermarket/commercial product
4.	Cheese(feta)	Hypermarket/commercial product
5.	Cheese	Hypermarket/commercial product
6.	Cheese(cascaval)	Hypermarket/commercial product
7.	Cheese	Hypermarket/commercial product
8.	Chesse	Hypermarket/commercial product
9.	Chesse	Hypermarket/commercial product
10.	Yougurt	Hypermarket/commercial product

The positive control (PC) sample was considered DNA extracted from cow’s muscle tissue, whereas the negative control (NC) was represented by a DNA sample of vegetal origin.

The DNA was extracted from 50 mg of raw material using InnuPREPDNA Mini Kit (Analytik Jena AG).

The DNA was evaluated for quality and quantity by the spectrophotometric method using the NanoDrop 8000 (ThermoScientific, USA) equipment, after the measurement the samples were brought to the same concentration by dilution.

PCR was carried out in final reaction volumes of 25 µl containing 100 ng of DNA template. The composition of amplification mixture was carried out according to instructions for DreamTaq Green PCR Master Mix (2X) commercial kit (Thermo Scientific, Lithuania). The following PCR program was used: 95° C for 5 min., 35 cycles programed as follows: 95°C for 30s, 55°C for 1 min., 72° C for 1 min. and the final extension at 72°C for 5 min.

Amplicons were run on 1.8 % agarose gels in TAE buffer at 100 V for 90 minutes.

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

The primers pairs used in this study are listed in Table 2

Table 2

Primer pairs used in this study

Species	Primers sequence forward/reverse 5'…3'
cow	GTACTACTAGCAACAGCTTA GCTTGATTCTCTTGGTGTAGAG
sheep	ATATCAACCACACGAGAGGAGAC TAAACTGGAGAGTGGGAGAT
goat	CGCCCTCCAAATCAATAAG AGTGTATCAGCTGCCAGTAGGGTT
buffalo	CTGTGTTCGCCATTATAGGA GTGGTTAGATCTACGGTTGAG

Results and discussions

Primarily in the study, total genomic DNA was purified from the samples considered in this study. The purified DNA was of good amplifiable quality ranging from 23 ng/µl to 58 ng/µl, depending on product texture, enabling the development of the further enzymatic reactions.Serial dilutions of DNA solution were prepared in order to equalize the genes copies number present in the DNA samples.

For the validation of PCR obtained data, a positive and a negative reaction controls were analyzed along with the samples in the same reaction conditions.

According to our determination, in samples 1, 2, 3 and 4 composition was in concordance with the label description of the product (Fig. 1, 2, 3, 4). Therefore, in the composition of analyzed dairy products the cow milk was detected, namely in cheese and sour cream prepared from cow milk.

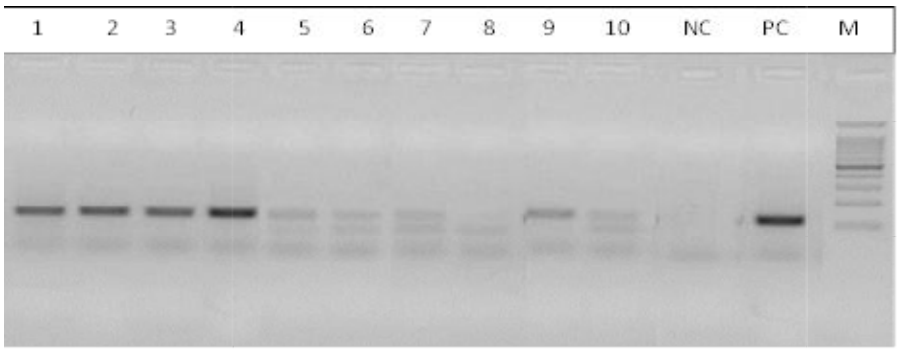

Fig. 1.PCR amplification for cow specie identification:
1. sample 1- raw milk ; 2. sample 2-3 –sour cream; 3. sample 4-9 cheese; 5. sample 10 - yogurt; NC – negative control; PC – positive control, DNA isolated from cow muscle tissue; M. DNA Ladder- PCR Marker (Promega)

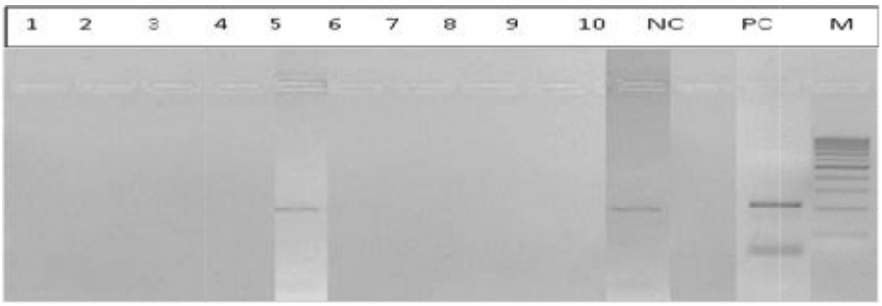

Fig. 2.PCR amplification for buffalo specie identification:
1. sample 1- raw milk ; 2. sample 2-3 –sour cream; 3. sample 4-9 cheese; 5. sample 10 yogurt; NC – negative control; PC – positive control, DNA isolated from cow muscle tissue

Those samples were acquired from the supermarket and are commercial products, except sample 1 who was a traditional product from a local farmers market. Our findings suggest that the compositions of the products that we analyzed are respecting the label description. Considering that by the same method, Hutu et al, 2013 and Mafra et al, 2004 were able to detect adulteration of dairy products, it can be said that the analyzed samples are free of adulteration.

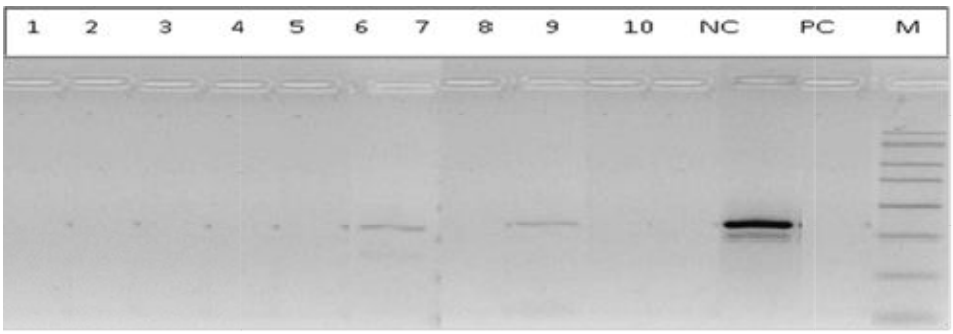

Fig. 3.PCR amplification for goat specie identification:
1. sample 1- row milk ; 2. sample 2-3 –sour cream; 3. sample 4-9 cheese; 5. sample 10 yogurt; NC – negative control; PC – positive control, DNA isolated from cow muscle tissue

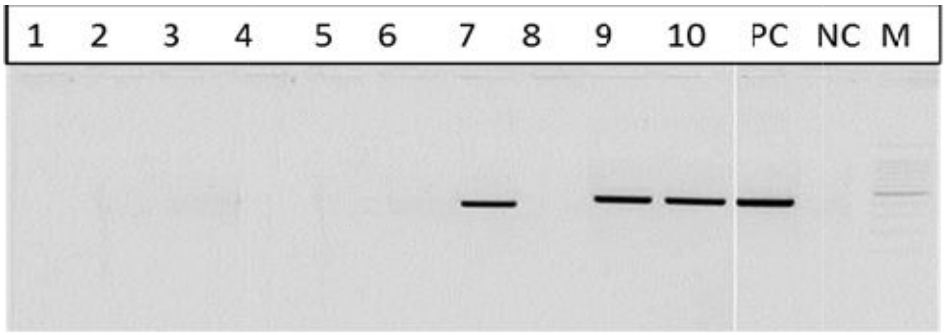

Fig.4.PCR amplification for sheep specie identification:
1. sample 1- row milk ; 2. sample 2-3 –sour cream; 3. sample 4-9 cheese; 5. sample 10 yogurt; NC – negative control; PC – positive control, DNA isolated from cow muscle tissue

In sample 5, we found buffalo cheese and the dairy product was the same with the label of the product. In samples 6, 8, 10 we found goat milk which was the same with the label description of the product.

In the last samples 7, 9 and 10 sheep milk it was detected and again our determination was according with the label of the product. In the end, all the samples were according with the label description of the product. However, the percentageof each detected specie in the composition of the dairy products it cannot be estimated because a PCR detection qualitative method was used in this study. A summary of the obtained results are summarized in Table 2.

Table 2

The composition of analyzed dairy products

Nr. Crt.	Biological sample	Label composition	Identified species
1.	Milk	Cow	Cow
2.	Sour cream 12% fat	Cow	Cow
3.	Sour cream 12% fat	Cow	Cow
4.	Cheese(feta)	Cow	Cow
5.	Cheese	Buffalo	Buffalo
6.	Cheese(cascaval)	Goat	Goat
7.	Cheese	Sheep	Sheep
8.	Chesse	Goat	Goat
9.	Chesse	Sheep	Sheep
10.	Yougurt	Goat and Sheep	Goat and Sheep

Conclusion

The duplex PCR method proposed in this study can be considered as a further improvement of a PCR based assay for the control of dairy products.

The specificity of the proposed primers pairs was proven by the validation experiment, along with the assay sensitivity and accuracy.

The obtained results pointed out the need for authorized controls on the market, especially in the case of local producers that are using the adulteration as a common practice.

The test could be useful in the control of dairy products, to verify the origin of the raw materials, especially in products submitted to denaturing technologies, for which other methods cannot be applied.

Acknowledgement

This work was conducted in Laboratories A1c and A2 from HoriaCernescu Research Unit of Banat's University of Agricultural Sciences and Veterinary Medicine „ King Michael I of Romania", Timisoara, Romania.

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NOTES ON SOMATIC CELLS COUNT IN DAIRY MASTITIS DETECTION

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Summary

The study was carried out during the fall season, in one dairy farm, on somatic cells count (SCC) collected from cows on the same day as milk samples for microbiology testing of mastitis. Transversal and retrospective analyses of SCC were carried on in order to observe a possible association between detection by SCC, California Mastitis Test positive (CMT+), milk quality indicators and microbiological mastitis. From a BIOAMR data basis, 60 cows subclinical and microbiological diagnosed with mastitis were sampled - in 70% of cases was identified *Staphylococcus aureus*. The SCC were retrospectively correlated with previous' milk urea nitrogen or MUN (r =+0.655, at p =0.001), SCCs' trend (r = + 0.815, at p =0.000) and transversal correlated with protein (r = + 0.342 at p =0.031), lactose (r = + 0.357 at p =0.024) and density (r = + 0.320 at p =0.044. The fifth classes of SCC was retrospectively associated with fat (p = 0.040), MUN (p = 0.022), and transversally associated with SCC trend (p = 0.000), freezing point (p = 0.060), number of glands CMT + (p =0.006), left front tits CMT + (p =0.027), and rear right tits CMT+ (p =0.060). The SCC was easily associated in retrospective or transversal approach of milk quality indicators to CMT, but the study cannot found the association between specific germs (including MRSA) of mastitis and SCC. On such condition, further study has to be developed in order to improve the detection more specifically in the presence of pathogen bacteria.

Keywords: somatic cells count, milk constituents, mastitis detection

In clinical mastitis, signs such as abnormal milk (changes in color, presence of clots, flakes), abnormal mammary gland (changes in tissue color, swelling) and changes in animal status (14) such body temperature (10), appetite, and hydration level, are easily visible. In subclinical mastitis the majority of previous signs are not detectable (1, 2). In both cases the somatic cells count can easily be monitored (12).

Somatic cell count (SCC) is the most common method for the detection of mastitis. If the detection is followed by diagnosing the bacterial agent, the results can help to determine treatment and prevention strategies on the farm, which in turn can help to reduce incidence and prevalence (1).

The aim of the study is a transversal and retrospective study of association of SCC with milk constituents in cases of animals *a posteoriori* diagnosed with pathogenic germs of mastitis.

Materials and methods

Farms and animals sampling: 20 partner farms of Extension unit from four counties of West Romania were stratified sampled (5 farms for each of the counties Arad, Bihor, Timis and Hunedoara) in a screening for dairy mastitis infection. All farms were included and follow the Official Control of Milk Production managed by regional Breed associations (7-9, 11*)*. From the sampled farms, 60 cases clinically and microbiologically diagnosed with mastitis of the infected cows' quarts of udders were included in the retrospective study the infected cows' quarts of udder.

Fig. 1. CMT & collecting the milk samples for primary analysis at the farms level

Detection of mastitis with California Mastitis Test and milk sampling for positive CMT cows at the milking parlor site (left). Animal Production Laboratory (right). Analyzing the milk CMT positive sample for milk constituents and content (Funke Gerber Lactostar Dairy Analyser) and somatic cells count (DeLaval cell counter DCC).
Source: UEX Media, 2019. The student team of Bioeconomic approach to antimicrobial agents - use and resistance project during the farm visits activities.

Data collection and processing: the Californian Mastitis Test (CMT) and milk samples have been taken and primarily analyzed (Figure no. 1) on the farm for all dairy cows. Only positive sample to CMT were collected for analyzing the chemical milk constituents (*Funke Gerber Lactostar Dairy Analyser*) and SCC analysis (*DeLaval cell counter DCC)*. The SNF (fat free dry matter), protein, fat, lactose and minerals with maximum ± 0.04 % reproducibility were measured, and freezing point and density were calculated.

Microbiology analysis of samples collected by C*OPAN's ESwab*^{TM} system was effectuated for all CTM positive quarters. Each infected quarter was considered an individual sample. The germs were isolated by classical microbiological exam. Commercial culture-based tests are available for diagnose of mastitis such us: CHROMagar Mastitis (CHROMagar, France), Hardy Diagnostics Mastitis Triplate (Hardy Diagnostics, USA), Minnesota Easy Culture System II Triplate (University of Minnesota, USA), and VétoRapid (Vetoquinol, the Netherlands) *(4).* The types of germs and antimicrobial resistance (AMR) were analyzed by *Walk Away System* using *MicroScan® Dried Panels.*

Statistical Analysis: *SPSS® Statistics* software for *Spearman's* correlation, *χ2 test, Mann-Whitney U test* were used in order to do the analyses of association, frequency and differences between SCC trend and several groups and variables of the study. The hypothesis was accepted at the lever of value α = 0.05.

Results and discussions

Milk from cows with mastitis cannot be used for consumption because it has altered chemical composition and organoleptic proprieties. In juridical prospect, the maximum level of CSS has to be maximum 400.00 x 10^3 cell/ml of milk. In the collected sample, the average value of SCC was more than three time mode - 1,426.18±244.99 x 10^3 cells/ml of milk. So, by the number of somatic cells it is clear that milk cannot be used for human consumption but, by diluted in the culling tank with normal milk, the average of SCC in milk tank decreases under legal limit. However, some studies have proven an increased prevalence of resistant bacteria from dairy animals undergoing antibiotic treatment (3). In practice using non-salable milk from cows under antibiotics treatment to feed the calves will increase the risk of increasing fecal shedding of AMR bacteria (13).

The SCC were retrospectively correlated with previous' milk urea nitrogen or MUN (r =+0.655, at p =0.001), SCCs' trend (r = + 0.815, at p =0.000) and transversal correlated with protein (r = + 0.342 at p =0.031), lactose (r = + 0.357 at p =0.024) and density (r = + 0.320 at p =0.044. The fifth classes of SCC was retrospectively associated with fat (p = 0.040), MUN (p = 0.022), and transversally associated with SCC trend (p = 0.000), freezing point (p = 0.060), number of glands CMT + (χ^2 =33.90 at p =0.006), left front tits CMT + (χ^2 =10.94 at p =0.027), rear right tits CMT+ (χ^2 =9.03 at p =0.060).

By transversal analysis, the positive bacterial samples (which were positive to bacteria) SCC was associated with sensibility and AMR to Clindamycin (χ^2 = 22.97 at p = 0.003), Linezolid (χ^2 = 11.66 at p = 0.020), Netilmicin (χ^2 = 17.94 at p = 0.001), Synercid (χ^2 = 26.30 at p = 0.001) and Vancomycin (χ^2 = 20.77 at p = 0.008). In retrospective analysis, the trend of SCC was associated with sensibility and AMR to Clarithromycin (χ^2 = 6.98 at p = 0.030), Clindamycin (χ^2 = 8.18 at p = 0.017), Erythromycin (χ^2 = 8.80 at p = 0.012), Linezolid (χ^2 = 6.98 at p = 0.033),

Netilmicin (χ^2 = 4.06 at p = 0.044), Synercid (χ^2 = 13.62 at p = 0.001) and Vancomycin (χ^2 = 8.88 at p = 0.012). In this case, the trend was associated with AMR detection for more times. In comparison with the transversal study, the retrospective index works much better.

The SCC was easily associated in retrospective or transversal approach (8) of milk quality indicators to CMT, but the present study did not find the association between specific germs (including MRSA) of mastitis and SCC. May be analyzing other biomarkers, such as released enzymes reflecting tissue destruction (*Lysosomal N-acetyl-β-d-glucosaminidase - **NAGase** or Lactate dehydrogenase - **LDH** or **LD***) will improve the association between detection and diagnosis of mammary gland inflammation (5, 6).

Conclusions

In terms of results provided by SCC detection, the dynamic or retrospective analysis works better than the transversal approach.

The study cannot found the association between specific germs (including MRSA) of mastitis and somatic cell count (SCC).

Acknowledgements

The activities were financially supported by project *Bioeconomic approach to antimicrobial agents - use and resistance*, Contract no. *7PCCDI, PN-III-P1-1.2-PCCDI-2017-0361* financed by *UEFISCDI* and research was run within Animal Production Laboratory, part of *Horia Cernescu* Research Unit in *Banat University of Agricultural Science and Veterinary Medicine "King Michael I"*, Timisoara.

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