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APPROACHING FOOD-BORNE PARASITES STATUS IN THE WORLD AND ROMANIA

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Key words: food-borne parasites, status, world, Romania

Summary

Food-borne parasitic zoonoses have a major impact on the health and economy, with an widespread in the context of actual changes of the complex socio-economic and socio-cultural factors. Globalization of the food supply, increased international travel, increase of the population of highly susceptible persons, change in culinary habits, but also improved diagnostic tools and communication are some factors associated with the increased diagnosis of food-borne parasitic diseases worldwide.

Zoonotic parasites found in food animals include a wide variety of protozoa, trematodes, cestodes, and nematodes. These food borne parasites reach the human beings through the consumption of raw infected food such as muscle tissues of different animal species (*Toxoplasma gondii, Sarcocystis hominis, Sarcocystis suishominis, Diphyllobotrium latum, Taenia solium, Taenia saginata, Opisthorchis felineus, Anisakis spp., Pseudoterranova spp., Trichinella* spp.), or vegetables (*Fasciola hepatica*), and contaminated food and water resources (*Giardia duodenalis, Cryptosporidium spp., T. gondii, Echinococcus granulosus* sensu latu, *Echinococcus multilocularis, T. solium, Taenia multiceps*). Meat of fish, reptiles and amphibians can be infected with a variety of parasites, including trematodes (*Opisthorchis* spp., *Clonorchis sinensis*, minute intestinal flukes), cestodes (*Diphyllobothrium* spp., *Spirometra*), nematodes (*Gnathostoma*, spp., anisakine parasites), and pentastomids that can cause zoonotic infections in humans when consumed raw or not properly cooked.

The current status of the main food-borne parasites, with registered prevalence of infection in different countries and areas of the world, and the economic losses resulting from this, is reviewed in the first part. In the second part, the situation of some zoonotic diseases registered in Romania, is presented.

The complex of effective measures for monitoring and control of food-borne parasites, including education of farmers, shepherds and consumers, improving of farming conditions, a control of sewage sludge on pastures and of drinking water resources, improved transportation and distribution systems of food, accompanied by a new technology in food processing, specialized inspection associated with a supplementary standardized surveillance, is needed to further reduce the incidence of these diseases.

Zoonotic parasites found in food animals include a wide variety of protozoa, nematodes, trematodes, and cestodes. More than 72 species of protozoan and helminth parasites can reach humans by food and water, and most of these infections are zoonoses (Pozio, 2003).

Many of these parasites are emerging or already occur globally due to changes in farming practices and the increased movement of animals, food, and people. The potential for global occurrence of these parasites is increasing (Gajadhar et al., 2006). Globalization of the food supply,

increased international travel, increase of the population of highly susceptible persons, change in culinary habits, but also improved diagnostic tools and communication are some factors associated with the increased diagnosis of food-borne parasitic diseases worldwide (Dorny et al., 2009).

Foodborne parasites can be divided in two main groups according to the way of transmission to humans. These foodborne parasites reach the human beings through the consumption of raw infected food such as *muscle tissues* of different animal species (*Toxoplasma gondii, Sarcocystis hominis, Sarcocystis suishominis, Diphyllobotrium latum, Taenia solium, Taenia saginata, Opisthorchis felineus, Anisakis spp., Pseudoterranova spp., Trichinella* spp.), or *vegetables* (*Fasciola hepatica*), and contaminated food and water resources (*Giardia duodenalis, Cryptosporidium* spp., *T. gondii, Echinococcus granulosus* sensu latu, *Echinococcus multilocularis, T. solium, Taenia multiceps*) (Pozio, 2008).

Some parasites show a cosmopolitan distribution, others a more restricted distribution due to their complex life cycles, which need the presence of one or more intermediate hosts.

Of this large number of pathogens, only *Toxoplasma gondii* can be transmitted to humans by two different ways, i.e., by cysts present in infected meat and by oocysts from the feces of infected cats, contaminating food and water. So, the potential exists for both waterborne and foodborne toxoplasmosis. This parasitic protozoa do not multiply in foods, but they may survive in or on moist foods for months in cool, damp environments. Their ecology makes control of these parasites difficult.

There are some recent indications that *Toxoplasma* infections acquired by adults by ingestion of sporulated oocysts may be more pathogenic than cyst-induced infections. In such cases, eye lesions are quite frequent and were previously thought to be predominantly acquired by prenatal infection (Dubey et al., 2005).

Until recently, waterborne transmission of *T. gondii* was considered uncommon, but a large human outbreak linked to contamination of a municipal water reservoir in Canada by wild felids and the widespread infection of marine mammals in the USA provided reasons to question this view (Jones J.L. and Dubey J.P., 2009).

Each year throughout the world, *Toxoplasma gondii* infects millions of persons, who contract it either by eating raw or poorly cooked meat from infected animals such as hogs or sheep or by ingesting soil contaminated with cat feces. About 30-50% of women of child-bearing

age are at risk of acquiring the infection during pregnancy with the potential of prenatal infection and severe disease of the foetus. In the United States between 400 and 10,000 infants are born each year with congenital toxoplasmosis. Toxoplasmic encephalitis, marked by dementia and seizures, has become the most commonly recognized cause of central nervous system opportunistic infection in AIDS patients (Schantz and McAuley, 1991).

Prevalence of viable Toxoplasma gondii in beef, chicken, and pork from retail meat stores, and risk assessment to consumers, was determined in the United States (Dubey et al., 2005). The prevalence of viable Toxoplasma gondii was determined in 6,282 samples (2,094 each of beef, chicken, and pork) obtained from 698 retail meat stores from 28 major geographic areas. To detect viable T. gondii, meat samples were fed to T. gondii-free cats and feces of cats were examined for oocyst shedding. In all, 7 cats fed pooled pork samples shed oocysts. Toxoplasma gondii oocysts were detected microscopically in the feces of 2 of the cats, and the remaining 5 pooled pork samples had so few oocysts that they were not initially detected by microscopic examination, but rather by mouse bioassay of cat feces. None of the cats fed chicken or beef samples shed oocysts. The prevalence of viable T. gondii in retail meat was very low. Nevertheless, consumers, especially pregnant women, should be aware that they can acquire T. gondii infection from ingestion of undercooked meat, and in particular, pork. Cooking meat to an internal temperature of 66°C kills T. gondii (Dubey et al, 2005).

The overall estimate of *Toxoplasma* seroprevalence in sheep from 16 districts distributed in seven areas of France, was 17.7% (11.6-31.5%) for lambs and 89% (73.5-100%) for adults ($P<0.0001$). No significant difference was observed between imported and French meat. In France, seroprevalence in lambs showed an increasing North-western to Southern gradient. The proportion of French carcasses carrying live parasites according to bioassay results was estimated at 5.4% (3-7.5%) (Halos et al, 2009).

In a study performed in West Germany, based on empirical data, it was estimated that 4% of the German population become infected per year by *Toxoplasma gondii* (Hinz, 1991).

A specific analyze indicates that toxoplasmosis frequently occurs in Africa, but the epidemiological patterns in these countries are far from being complete (Pozio, 1991). Food habits, presence or absence of domestic and/or synanthropic felines, and environmental characteristics

(damp or dry areas) seem to influence the prevalence of infection in man from 15 to over 60%.

In a study of toxoplasmosis in South Africa, there was an overall prevalence of 21% (2,147/10,228). Prevalence vary between the different cultural groups and from one geographical region to another. The prevalence rate for people of Namibia and Botswana was 9% (65/725) compared to the 30% (190/635) found in the Indian and Black communities of Kwazulu-Natal province, South Africa. These variations are probably linked to the dietary habits of the different cultural communities (Joubert and Evans, 1997).

Other protozoan parasites, *Cryptosporidium*, *Giardia*, and *Cyclospora* have proven potential to cause waterborne and foodborne disease.

Nowadays, the parasitic zoonose organisms *Cryptosporidium* spp. and *Giardia lamblia* are among the most relevant pathogens of drinking water-associated disease outbreaks (Dawson, 2005). These pathogens are transmitted via a fecal-oral route; in both cases the dose of infection is low. Apart from person-to-person or animal-to-person transmissions, the consumption of contaminated food and water are further modes of transmission. The disease is mainly characterized by gastrointestinal symptoms (Exner and Gornik, 2004).

Cryptosporidiosis outbreak investigations and analytic studies have associated the disease with drinking water supplies, animal contact, travel, and swimming pools. Contamination of the source water for drinking water supplies, as well as inadequate water treatment can be responsible for cryptosporidiosis outbreaks. Water-borne pathogens such as *Cryptosporidium* pose a significant human health risk and catchments provide the first critical pollution 'barrier' in mitigating risk in drinking water supply.

Cryptosporidium, *Giardia*, and *Cyclospora* have potential significance in the preparation and consumption of fresh produce and in catering practice, in which ready-to-eat foods may be served that have not received heat treatment. None of the three organisms *Cryptosporidium*, *Giardia*, and *Cyclospora* has been shown to be a problem for heat processed food or tap water that has undergone appropriate treatment at a water treatment works. All three are sensitive to standard pasteurisation techniques.

Cryptosporidium causes diarrhoeal disease that can be particularly severe in immuno-compromised individuals. Cryptosporidiosis is a notifiable disease at European Union level, and surveillance data are

collected through the European Basic Surveillance Network (Semenza and Nichols, 2007).

The disease distribution in Europe for 2005 showed 7,960 cryptosporidiosis cases reported from 16 countries. The crude incidence rate was 1.9 cases per 100,000, although there were considerable differences in the rates of cryptosporidiosis between countries. Infection was more commonly reported in young children. A pronounced seasonal peak was observed in the autumn of 2005, with 59% of the cases reported between August and November, although Ireland and Spain experienced a peak in spring and summer, respectively. Routine cryptosporidiosis surveillance from North West England over 17 years showed that the cases occurred predominantly in spring and autumn. British drinking water regulations and improvements in drinking water treatment have coincided with a decline in cryptosporidiosis incidence. Improvements in cryptosporidiosis surveillance such as detection, recording and reporting will help to recognize outbreaks and monitor interventions (Semenza and Nichols, 2007).

The first cases of giardiasis and cryptosporidiosis outbreaks registered in Germany in 2001, associated with drinking water, were reported by Exner and Gornik (2004). They asserted that in industrialized countries, the prevalence rate of giardiasis is 2-5 % and of cryptosporidiosis 1-3%.

The possibility of endemic transmission of water-borne protozoan diseases in some areas from Mongolia was reported (Huh et al., 2006). A total of 10 inhabitants (6.1%) from 165 residents, in Dornod and Selenge, Mongolia, were found to be infected with protozoan cysts or oocysts by stool examinations; 7 (4,2%), 1 (0,6%) and 2 cases (1.2%) with *Entamoeba coli*, *Giardia lamblia* and *Cryptosporidium parvum*, respectively.

Coexisting intestinal protozoa *Giardia lamblia* (5.2 and 4.9%) and *Entamoeba coli* (6.9 and 6.5%) were found also in a small fishing community on the Nam Ngum reservoir (Lao Republic) (Sithithaworn et al., 2006).

Non-dairy calves were identified as the major remaining source of *Cryptosporidium* in some regions from South Australia (Myponga catchment). The restriction of watercourse access of non-dairy calves could achieve a further reduction in *Cryptosporidium* export to the Myponga reservoir of around 90% from current levels (Bryan et al., 2009).

Giardia and *Cryptosporidium* are detected in surface water and sporadically in unprotected groundwater. Use of these waters for

drinking water abstraction makes high demands on the technology of the treatment process: because of the disinfectant resistance of the parasites, safe elimination methods are needed, which even at high contamination levels of source water guarantee safe drinking water.

Further measures for prevention and control are implementation of the HACCP concept, which includes the whole chain of procedures of drinking water supply from catchments via treatment to tap and a quality management system (Exner and Gornik, 2004).

Food-borne trematodiases pose a significant public health and economic problem, but these diseases are still often neglected. The changing epidemiological pattern and the rapid growth of aquaculture and food distribution networks are highlighted, as these developments might be associated with an elevated risk of transmission of food-borne trematodiases. An estimated 750 million people are at risk of infections with food-borne trematodes, which comprise liver flukes (*Clonorchis sinensis, Fasciola gigantica, Fasciola hepatica, Opisthorchis felineus, and Opisthorchis viverrini*), lung flukes (*Paragonimus* spp.), and intestinal flukes (*Echinostoma* spp., *Fasciolopsis buski*, and the heterophyids) (Keiser and Utzinger, 2009).

Ones of these trematodes such as *Clonorchis* and *Paragonimus*, which are transmitted via fish or crustaceans and cause serious human disease in specific regions of the world.

Foodborne trematodiasis is an emerging public health problem, particularly in Southeast Asia and the Western Pacific region (Keiser and Utzinger, 2005). Currently, 601 million people are at risk for infection with *Clonorchis sinensis*, 293.8 million with *Paragonimus* spp., 91.1 million people with *Fasciola* spp., and 79.8 million with *Opisthorchis* spp. Residents living near freshwater bodies have a 2.15-fold higher risk (95% confidence interval 1.38-3.36) for infections than persons living farther from the water. Exponential growth of aquaculture may be the most important risk factor for the emergence of foodborne trematodiasis. This is supported by reviewing aquaculture development in countries endemic for foodborne trematodiasis over the past 10-50 years (Keiser et Utzinger, 2005).

Fascioliasis and other food-borne trematodiases are included in the list of important helminthiases with a great impact on human development. Six plant-borne trematode species have been found to affect humans: *Fasciola hepatica, Fasciola gigantica* and *Fasciolopsis buski* (Fasciolidae), *Gastrodiscoides hominis* (Gastrodiscidae), *Watsonius watsoni* and *Fischoederius elongatus* (Paramphistomidae).

Whereas *F. hepatica* and *F. gigantica* are hepatic, the other four species are intestinal parasites.

The fasciolids and the gastrodiscid cause important zoonoses distributed throughout many countries, while *W. watsoni* and *F. elongatus* have been only accidentally detected in humans.

Present climate and global changes appear to increasingly affect snail-borne helminthiases, which are strongly dependent on environmental factors. Fascioliasis is a good example of an emerging/re-emerging parasitic disease in many countries as a consequence of many phenomena related to environmental changes as well as man-made modifications. The ability of *F. hepatica* to spread is related to its capacity to colonise and adapt to new hosts and environments, even at the extreme inhospitality of very high altitude. Moreover, the spread of *F. hepatica* from its original European range to other continents is related to the geographic expansion of its original European lymnaeid intermediate host species *Galba truncatula*, the American species *Pseudosuccinea columella*, and its adaptation to other lymnaeid species autochthonous in the newly colonised areas.

In West Germany, there are only sporadic cases of fascioliasis, according to eating habits (Hinz, 1991).

Fasciolopsiasis has become a re-emerging infection in recent years and gastrodiscoidiasis, initially supposed to be restricted to Asian countries, is now being reported in African countries (Mas-Coma et al., 2005). Although fasciolopsiasis and gastrodiscoidiasis can be controlled along with other food-borne parasitoses, fasciolopsiasis still remains a public health problem in many endemic areas despite sustained WHO control programmes.

Fish- or crustacean-borne trematodes (species of *Clonorchis*, *Opisthorchis*, *Paragonimus*, intestinal flukes) infect about 39 million people, and about 550 millions are at risk (WHO, 1995). Traditionally, these parasitic zoonoses are most common in Asia because of the particular food practices and the importance of aquaculture. However, some of these parasites may emerge in other continents through aquaculture and improved transportation and distribution systems. Human paragonimiasis is present in Western Africa with a prevalence ranging from 2 to 31% (Pozio, 1991).

Parasitological surveys in Xai Udom, a small fishing community on the Nam Ngum reservoir (Lao Republic), revealed an overall parasitic infection rate in May 1999 of 68.8% (n = 173) and in December 1999 of 65.9% (n = 261). The liver fluke, *Opisthorchis viverrini* accounted for most of the infections (prevalences of 53.8% and 42.1%, during the first

and second surveys, respectively). The prevalence and intensity showed increasing trends with age. Minute intestinal flukes were also present but with relatively low infection rates (3.8-10.9%) (Sithithaworn et al., 2006).

MEAT-BORNE PARASITE INFECTIONS

Humans get infected by eating raw or undercooked meat infected with cyst stages of specific parasites.

Meat inspection is the principal method applied in the control of *Taenia* spp. and *Trichinella* spp. However, it is often not very sensitive, frequently not practised, and not done for *T. gondii* and *Sarcocystis* spp.

Meat of reptiles, amphibians and fish can be infected with a variety of parasites, including trematodes (*Opisthorchis* spp., *Clonorchis sinensis*, minute intestinal flukes), cestodes (*Diphyllobothrium* spp., *Spirometra*), nematodes (*Gnathostoma*, spp., anisakine parasites), and pentastomids that can cause zoonotic infections in humans when consumed raw or not properly cooked.

While many in developed countries will recognize meat-borne zoonoses such as trichinellosis and cysticercosis, far fewer are acquainted with the fish-borne parasitic zoonoses which are mostly helminthic diseases caused by trematodes, cestodes and nematodes. These zoonoses are responsible for large numbers of human infections around the world. The list of potential fish-borne parasitic zoonoses is quite large. However, emphasis has been placed on liver fluke diseases such as clonorchiasis, opisthorchiasis and metorchiasis, as well as on intestinal trematodiasis (the heterophyids and echinostomes), anisakiasis (due to Anisakis simplex larvae), and diphyllobothriasis (Chai et al., 2005).

Other zoonotic infections are less frequent but may cause severe and lethal diseases, for example *Taenia solium* cysticercosis.

Cysticercosis infection in swine has almost disappeared in the Mediterranean area, while it is still present in some African countries (Pozio, 1991). Cysticercosis appears to be most prevalent in the Eastern Cape Province (former Transkei), where pigs roam freely and sanitation facilities are inadequate or non-existent. Segments of tapeworms often feature as an ingredient of concoctions prepared by traditional healers and are suspected sources of many of the cases of cysticercosis in South Africa (Joubert and Evans, 1997).

Neurocysticercosis, caused by larvae of the pork tapeworm *Taenia solium*, is diagnosed in hundreds of persons in the United States every

year. Nearly all patients are immigrants or travelers from Mexico and other disease-endemic areas (Schantz, and McAuley, 1991). A total of 1,494 patients with neurocysticercosis were reported in the United States among large case series (n > 20) between 1980 and early 2004. Common onset symptoms for these patients included seizures (66%), hydrocephalus (16%), and headaches (15%). The majority presented with parenchymal NCC (91%), with the remainder having ventricular cysts (6%), subarachnoid cysts (2%), and spinal cysts (0.2%) (Wallin and Kutrzke, 2004).

Intestinal taeniid tapeworm infection acquired in the United States is almost entirely caused by *Taenia saginata*, the beef tapeworm (Schantz, and McAuley, 1991).

Bovine cysticercosis is an important food safety issue in Europe, and is of economic concern, too. In spite of the EU directives that regulate meat inspection for bovine cysticercosis, *Taenia saginata* is still present in Europe and causes economic losses due to condemnation, refrigeration and downgrading of infected carcasses. The main reasons for this persistence include the low sensitivity of current meat inspection protocols, the dissemination and survival of eggs in the environment and cattle husbandry systems, which allow grazing on pastures and drinking from water streams. It is assumed that water streams and surface water are potentially contaminated with *T. saginata* eggs. Furthermore, current wastewater management not only fails to halt, but rather contributes to the dissemination of eggs in the environment. Nowadays, the authors discuss an integrated approach for control of this food-borne zoonosis, as well as the potential use of serological methods as a way of improving detection of bovine cysticercosis (Dorny and Praet, 2007).

In Belgium, in the last years an increase in the number of bovine cysticercosis cases, mostly light infections, was observed. The role of contact with contaminated surface water has been hypothesized as the main route of transmission. A logistic regression analysis revealed that the location (province), the number of slaughtered cattle, the flooding of pastures, free access of cattle to surface water and the proximity of wastewater effluent were significant explanatory variables for bovine cysticercosis to be recorded in a herd (Boone et al, 2007).

A cross sectional study on *Taenia saginata* cysticercosis was carried out in slaughtered cattle in Iran in order to determine the infection rate during a three-years period, from 2005 to 2007. A total of 4,534,105 cattle were examined by routine meat inspection. The results showed that 11,410 cattle (0.25 %) were infected with *Cysticercus bovis*; among those 1,041 carcasses (0.02%) were condemned. In such carcasses the

metacestodes caused extensive damage in the vicinity of cysts in infected cattle. The rejected carcasses had an average of 410 thousands USD loss annually (Jahed et al, 2009).

Cysticercosis infection in cattle is widespread in Africa with a prevalence ranging from 2 to 50% in relation to breeding and human habits; in European countries of the Mediterranean area the prevalence of infection is below 2% (Pozio, 1991).

In parasitological surveys in some Asian areas (Xai Udom, a small fishing community on the Nam Ngum reservoir, Lao Republic), showed concurrent tapeworm infections: *Taenia* (1.7 and 1.1%) and *Hymenolepis nana* (0.7 and 0.6%) (first and second surveys) (Sithithaworn et al., 2006).

Another important zoonotic meat-borne disease is trichinelosis which is widespread in the world.

Although, in the United States trichinellosis was associated historically with eating *Trichinella*-infected pork from domesticated sources, wild game meat was the most common source of infection during 1997-2001. During this 5-year period, 72 cases were reported to CDC. Of these, 31 (43%) cases were associated with eating wild game: 29 with bear meat, one with cougar meat, and one with wild boar meat. In comparison, only 12 (17%) cases were associated with eating commercial pork products, including four cases traced to a foreign source. Nine (13%) cases were associated with eating non-commercial pork from home-raised or direct-from-farm swine where U.S. commercial pork production industry standards and Regulations do not apply (Roy et al, 2003).

Since 1947, when the US Public Health Service began to record statistics on trichinosis cases in humans, the numbers of reported cases in the United States have declined markedly, from an average of about 400 with 10-15 deaths reported each year in the late 1940s, to an average of 57 per year with three deaths overall in the 5 years 1982-1986 (Schantz and McAuley, 1991).

The majority of the decline in reported trichinellosis cases in the USA is a result of improved observance of standards and regulations in the U.S. commercial pork industry, which has altered animal husbandry practices resulting in reduced *Trichinella* prevalence among swine. Because of the change in epidemiology of trichinellosis and the continued occurrence of cases among consumers of wild game meat and non-commercial pork, more targeted public education is needed to further reduce the incidence of this disease (Roys et al, 2003).

Sylvatic trichinellosis is widespread in Mediterranean (Trichinella sp.3) and African (*T. nelsoni, Trichinella* T8) regions. Domestic trichinellosis (*T. spiralis spiralis*) is present in Spain, France, Yugoslavia, Egypt, Gambia, and Nigeria in domestic and/or sylvatic animals. In Africa human trichinellosis is rare, mostly from religious and food habits. Till now very few control projects against food-borne parasitic zoonoses have been developed in Africa (Sithithaworn et al., 2006).

Diphyllobothriasis and **anisakiasis** both have increased in recent years in association with increasing popularity of raw fish dishes. Adequate prevention and control of food-borne parasitic zoonoses require continued and improved programs to educate consumers, producers and medical practitioners (Schantz and McAuley, 1991).

In West Germany, there are only sporadic cases of anisakiasis, according to eating habits (Hinz, 1991).

A few parasites that may be transmitted through faecal contamination of foods and that have received renewed attention, such as *Toxoplasma gondii*, or that are (re-)emerging, such as *Trypanosoma cruzi* and ***Echinococcus*** spp.

Affecting man and majority species of mammals, echinococcosis/ hydatidosis, caused by the larval stage of *E. granulosus*, is a serious zoonotic parasitosis which causes severe public health problems and important economic losses. Echinococcosis / hydatidosis is overspread in all continents, with a high prevalence in Mediterranean areas, the former Soviet Union, China, the North-East of Africa, Australia, and South America (Eckert et al., 1996, 2001). The presence of this parasite coincide with high prevalence of human cystic echinococcosis. The most affected regions are the Mediterranean basin (especially parts of Spain, southern Italy, and Sardinia, where annual incidence rates in human reach 4-8/100,000), and the sheep raising areas of Great Britain (Eckert et al., 2001).

Despite the big efforts that have been made in research and control of the E/H, this disease still remains one of the most important worldwide zoonosis. This is due to various factors, the most important being the close association between man, sheep, and dogs, in areas where open farming is practised.

There are reports on re-emerging of E/H in some regions, where the prevalence of E/H was low (e.g. Cypru). Also, disturbing of the trends of the *E. multilocularis* distribution, with increasing of the detection rates of infestations in Europe and increasing of the high infected communities in China were registered (Torgerson and Budke, 2003).

The outbreaks of hydatidosis in swine were reported in other countries: 6.8% in a region of China; 5.16% (130 of 2500) in a slaughtered lot from Poland; 36%, 51.2% and 82.5% respectively, in three outbreaks in Mexic.

The global economic losses associated with human cystic echinococco-sis were estimated at US $ 763,980,979. When corrected for underreporting, annual losses were estimated at US $ 1,918,318,955 (Budke et al, 2006).

Livestock cystic echinococcosis associated economic losses were evaluated, and minimal annual losses, assuming just liver condemnation with no correction for underreporting, were estimated at US $ 141,605,195. However, when losses from additional production factors (decreased carcass weight, decreased milk production, decreased hide value, decreased fecundity) were taken into account, losses range from US $ 1,249,866,660, not taking into account underreporting, up to US $ 2,190,132,464 (Budke et al., 2006).

An other common group of parasites comprises soil-transmitted nematodes, such as *Trichuris trichiura*, *Ascaris lumbricoides*, hookworm and *Strongyloides stercoralis*, which are identified mostly in country or areas/communities with poverty and lack of hygiene.

In Romania the most frequent food-borne parasitic diseases reported are toxoplasmosis, cryptosporidiosis, trichinelosis and hydatidosis. Other sporadic zoonotic food-borne parasitosis are fasciolosis,

A high incidence of parasitic infections was shown by the study of children with physical and psychic handicaps. In 231 children examined were diagnosed 294 parasitic infections as follows: 42 (18.1%) with *Giardia intestinalis*, 6 (2.59%) with *Entamoeba histolytica*, 36 (15.5%) with *Hymenolepis nana*, 21 (9.9%) with *Strongyloides stercoralis*, 16 (6.9%) with *Ascaris lumbricoides*, 92 (39.86%) with *Tricocephalus dispar*, 38 (16.4%) with *Enterobius vermicularis*, 7(3.03%) with *Cryptosporidium* sp. Also, by immunodiagnosis 29 cases of Toxoplasmosis (12.5%) and 7 cases of Toxocarosis (3.03%) were pointed out. The clinical examination revealed that the main symptoms were troubles of appetite (both anorexia and hyperorexia) - 67.9%, intestinal transit disorders - 48.4%, cutaneous rash - 4.7%. Two series of specific treatments diminished the incidence at 38% (after the first) and at 28.5% (after the second) (Panaitescu et al, 1995).

To determine the prevalence of intestinal parasitic infections in 92 Romanian children institutionalized at Colentina Hospital and at the Dystrophic Center (Vidra, Romania). At least one protozoan was

identified in 77% of the fecal specimens examined. *Giardia lamblia* (72% of cases), *Cryptosporidium parvum* (12%), and *Entamoeba coli* (4%) were the only parasites identified. Protozoal colonization of the intestinal tract is common in institutionalized Romanian children and may play a role in causing morbidity and mortality in this high-risk group of children (Brannan et al, 1996).

In a retrospective statistic research in Romania based on the clinical survey and periodic control of a lot of 135 patients with toxoplasmosis, out of which 90 presented ocular toxoplasmosis (Teodorescu et al, 2008).

Toxoplasmosis is reported to induce granulomas in bone marrow inmunosuppressed patients. On the other side, long-term unexplained remissions after conventional treatment in multiple myeloma were mentioned in up to 10% of cases (Gologan et al, 2003).

A study was realized to get a real image about *Toxoplasma gondii* infection of pregnant woman and the consequence for her child in Moldavia area. The following results were obtained: 1) a high sero-prevalence of *T.gondii* antibodies among pregnant women (43.9%) - most of them being chronic infections; 2) 0.6% pregnant women with acute toxoplasmosis in the first trimester of their pregnancies, situation with great danger for the unborn child; 3) a 7.1% degrees of participation of *T. gondii* infection to the etiology of spontaneous abortion; 4) a high seroprevalence of *T. gondii* antibodies among children with mental retardation (66.4%) and visual pathology (37.4%) comparing with the group of apparently healthy children (9.3%). The conclusion resulting from this data is that toxoplasmosis demands more attention from our medical world, a national program of prophylaxis including a large screening of pregnant women and/or newborns being able to prevent the severe damages due to congenital toxoplasmosis (Cruceresc u, 1998).

The frequency of *Toxoplasma gondii* infection in the meat provided by two abattoirs, as well as the pathogenicity of the isolated strains were studied. The parasite carriage was investigated on 299 pools of diaphragmatic muscle (1 pool=10 animals) from 740 swine, 910 cattle and 1340 sheep: the methods used were bioassays on mice and the precocious identification of the tachyzoites in the peritoneal exudates and after 30 days the cerebral cysts.There were obtained 27 positive results (9.01%), in the examined pools of meat, without significant differences as concerns the frequency in relation to the animal species. Out of these strains two were virulent for mice and rabbits (Pop et al, 1989).

Twelve *Cryptosporidium* positive cases were identified, meaning a general prevalence of 2.48%, analyzing 481 faeces samples from patients -children and adults - presenting acute or prolonged gastroenteritis (Lazar and Radulescu, 1989). *Cryptosporidium* was found in 3.2% (4 of 123 children) on hospitalized children with acute diarrheal disease and bacterial and rotaviral infections (Constantiniu et al., 1991).

Cryptosporidiosis prevalence was 1.8% in a study group consists of 167 HIV serum-positive nonmaternally infected children (Cojocaru and Cojocaru, 1998).

The first report of human and pig trichinellosis in Romania dates back to 1868. After the political changes of 1989, the annual incidence increased from between 0.1 and 4.1 cases per 100,000 inhabitants during the communist period (1963-1989) to 6.2 cases per 100,000 inhabitants, with a range of 2-15.9 per 100,000 between 1990 and 2007. Trichinellosis is a major public health issue in Romania that requires that policies be put forth to advance efficient prevention and control strategies (Neghina et al., 2009a).

Retrospective analysis of the medical records of 335 patients found to have trichinellosis during 1996-2006 and hospitalized in Arad County, the majority (64.8%) were inhabitants of the rural areas. Winter was the season with the highest number of cases (71.6%) (Neghina et al., 2009b). A study conduced a retrospective investigation of the incidence of human trichinellosis in Timis County over a period of 16 years (1990-2005), on 521 patients, revorded the highest number of cases in 1994 (16.90%) (Neghina et al, 2009c).

T. spiralis and *Trichinella britovi* are the only two species identified in a study conducted to identify *Trichinella* spp. circulating among wild and domestic animals in Romania using PCR-based methods. *T. spiralis* was the predominant species found in domestic animals (n=9; 75%), while *T. britovi* was more prevalent in wildlife (n=24; 86%) (Blaga et al, 2009).

Echinococsis/hidatidosis (E/H) has a high prevalence in animals and human, in Romania.

In a report of registered cases in the 2001-2004 period, the next values were found: - in bovine, 18.98% (from 859.162 slaughtered animals), ranging from16.21 to 20.97%; - in sheep, 12.65% (of 594.638 slaughtered animals), ranging from 8.29 to 14.5; - in swine, 3.81% (of 8,154,611), varying from 3.35 to 4.08%; - in horses, 12.12% (from 26,545 slaughtered animals), 4.02-24.32% (Ionescu, 2005).

A high prevalence of hydatidosis (ranges between 12-32.7%) in swine slaughtered in an abattoir from the South of Romania, during of 1992-1999 period, was registered (Mitrea et al, 2003).

In **Romania**, after an evaluation of the economic losses during of 1994-2004 period, was noticed that important organs condemnations were made because of cystic echinococcosis, totalizing 80.5 billion lei (Iacobiciu et al., 2005). Therefore, during of 1994-2004, in Mehedinti county, 59.9 ton of viscera with hydatidosis from cattle, sheep and pigs were confiscated, the annual condemnations varying between 5.9 and 13.6 ton. In Dolj county, during of the 1992-1995 period, 653 ton of infested viscera from cattle, sheep and pigs were confiscated, with an annual variation from 122 to 220 ton (Siko and Bokor, 1991).

The incidence of *E. granulosus* in dogs, which are the definitive host of the parasite, is very different, depending of the studied area, and animal category. Thus, the average of infestation was 15.4% (during of 1956-1997 period); the highest values was registered in straight dogs, followed by shepherd dogs (38.9%), and guarding dogs (18.5%) (Iacobiciu et al, 2005).

The average of hydatidosis prevalence in human, in Romania, is 5/100,000 habitants, varying from 0.24 to 7.2 (Gherman, 2000). V. Stefanoiu (1999) stated that more than 45% from the localities in Romania had at least one surgery case of hydatidosis.

In addition to human disease, some of these parasites are responsible for economic loss to livestock production.

Economic losses resulting from food-borne parasitic zoonoses are difficult to assess. Estimating the global economic impact of these diseases is handicapped by inadequate information on the prevalence and public health importance of parasitic zoonoses for most countries. However, the economic losses caused by certain zoonoses has been estimated for some regions and in these instances the costs are significant (Murrell K.D., 1991).

The public health and economic impact of meat- and fish-borne parasitic zoonoses is considerable in terms of morbidity and even mortality in humans as well as in losses due to reduced productivity in animals and condemnation of parasitized meat and fish. In this context, the increasing demands of consumers for meat and fish free of pathogens and chemical residues has to be considered.

CONCLUSIONS

The increased demand in animal proteins for human consumption will lead to an intensification of the production systems in which the risk of zoonotic parasitic diseases needs to be assessed.

The complex of effective measures for monitoring and control of food-borne parasites, including education of farmers, shepherds and consumers, improving of farming conditions, a control of sewage sludge on pastures and of drinking water resources, improved transportation and distribution systems of food, accompanied by a new technology in food processing, specialized inspection associated with a supplementary standardized surveillance, is needed to further reduce the incidence of these diseases.

Control of zoonotic parasites at the producer level requires education and the development and implementation of effective measures to eliminate the contamination of agricultural water and feed with viable stages of parasites. Use of contaminated waters for drinking water abstraction makes high demands on the technology of the treatment process: because of the disinfectant resistance of the parasites, safe elimination methods are needed, which even at high contamination levels of source water guarantee safe drinking water. Further measures for prevention and control are implementation of the HACCP concept, which includes the whole chain of procedures of drinking water supply from catchments via treatment to tap and a quality management system.

Overall, there is an urgent need for better monitoring and control of food-borne parasites using new technologies. For general control of parasitic species in the food chain, the following steps are necessary: - follow good hygienic practice in food service and catering industries; - minimise dissemination of parasitic stages (cysts, oocysts, eggs) in the farming environment and via human waste management; - include these organisms in Hazard Analysis Critical Control Point (HACCP) plans of water suppliers, industries or sectors that use fresh produce, and operations in which contaminated process or ingredient water could end up in the product (e.g., where water supplies may become contaminated) (Dawson, 2005).

The control strategies should include also the improvement or the development of more sensitive methods to detect these parasites in slaughtered animals and in foodstuff, and the reduction of contacts between livestock and wild animals which frequently represent the most important reservoir of these pathogens (Pozio, 2008).

Meat inspection is the principal method applied in the control of some zoonotic parasites, such as *Taenia* spp. and *Trichinella* spp. However, it is often not very sensitive, frequently not practised, and not done for *T. gondii* and *Sarcocystis* spp. Meat inspection of reptiles, amphibians and fish can avoid other zoonotic parasites, including trematodes (*Opisthorchis* spp., *Clonorchis sinensis*, minute intestinal flukes), cestodes (*Diphyllobothrium* spp., *Spirometra*), nematodes (*Gnathostoma*, spp., anisakine parasites), and pentastomids, that can cause zoonotic infections in humans when consumed raw or not properly cooked.

Of particular concern in industrialised countries is the increased travel and immigration, which increase the exposure to exotic diseases. In this condi-tions, standardisation, implementation, and documentation of control measures should increase confidence in global food trade (Gajadhar et al., 2006).

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MOLECULAR IDENTIFICATION OF SMALL STRONGYLE (*STRONGYLIDA: CYATHOSTOMINAE*) SPECIES USING A PCR-BASED REVERSE LINE BLOT HYBRIDIZATION ASSAY

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Key words: Reverse Line Blot hybridization, strongyles, cyathostomins, horses

Summary

A PCR-based Reverse Line Blot (RLB) hybridization assay has been applied for molecular identification of equine strongyle species in a farm from Central Kentucky (USA), using eggs and larvae. Strongyle eggs were harvested from feces from naturally infected horses; larvae were obtained from coprocultures in the laboratory. Additionally, four strongyle adults, morphological identified, were included as positive controls in the assay. DNA was extracted from eggs in groups of thousands and larvae in group of seven. The ribosomal DNA intergenic spacer was amplified by the polymerase chain reaction using primers derived from conserved regions within the flanking 18S and 26S rRNA genes. The PCR-amplicons were used for a non-radioactive hybridization with 13 species-specific oligonucleotide probes, and one genera-specific oligoprobe (for *Strongylus*). Nine cyathostomin species were molecularly identified by RLB assay: *Cylicocyclus nassatus, Cylicocyclus insigne, Cylicocyclus leptostomum, Cylicocyclus ashworthi, Cylicostephanus longibursatus, Cylicostephanus goldi, Cylicostephanus calicatus, Cylicostephanus minutus* and *Coronocyclus coronatus*. The results of this study show the efficacy of the PCR-RLB assay to determine distribution and species-specific occurrence of cyathostomins under field conditions and to discriminate them from the large strongyles, indicating an invaluable way to furthering drug-resistance studies.

Equids can harbor over 100 species of internal parasites (Krecek et al, 1987). About one-half of these species are in the strongyle group, the great majority (64 of 83 species) being members of a single family, the *Strongylidae* (Lichtenfels et al, 2008).

The equine strongyles (Nematoda: *Strongylidae*) consist in a large group of intestinal parasitic nematodes with relevant importance in equine clinical practice. The Strongylidae of horses includes two subfamilies: *Strongylinae* ("large strongyles") and *Cyathostominae* ("small strongyles").

The large strongyle group (strongylins) in horses historically was composed of three species in the genus *Strongylu*s (*S. vulgaris, S. edentatus,* and *S. equinus*). In the actual classification 14 species of Strongylinae of domestic equids are organized in 5 genera (Lichtenfels et al., 2008). One important characteristic of larval stages, especially of *S. vulgaris,* is that they migrate into blood vessels. This can result in

occlusion of the blood vessels and periodic colic; even death of infected horses may occur. However, following the introduction of ivermectin in 1983, which is highly effective against migrating larval stages of *Strongylus* spp., a further and dramatic reduction in the prevalence and intensity of *Strongylus vulgaris* occurred (Herd et al., 1990). As a result, *S. vulgaris* is no longer considered an important cause of colic in managed horses and is uncommonly diagnosed except on farms where parasite control is severe neglected (Kaplan, 2002).

Therefore, the decline of *S. vulgaris* and the rise of drug-resistant cyathostomes have changed the view of the relative importance of these nematodes; cyathostomes are now considered the principal parasitic pathogens of horses (Herd, 1990; Love et al., 1999; Uhlinger, 1990).

The cyathosomes, also called cyathostomins or small strongyles, includes 50 species worldwide (Lichtenfels et al, 1998). Virtually, 100% of horses are infected with at least some species of small strongyles (Reinemeyer et al, 1984). Infection with cyathostomins is complex and produces an inflammatory enteropathy which results in impaired intestinal microcirculation and motility (Love et all, 1999). Clinically, infection with adult cyathostomins can cause mild disease symptoms such as intermittent diarrhea, weight loss, poor hair coat, poor appetite, and lethargy with disordered intestinal motility, loss of condition, and peripheral edema (Love et al, 1992a,b; Matthews and Morris, 1995). Larval cyatostominosis is characterized by severe diarrhea, protein loss, enteropathy, and weight loss, especially as a result of emergence of massive numbers of larvae from the lining of the large intestines (Love et al, 1999).

The major challenges to understanding and controlling these parasites are the species complexity of the nematode populations, the inability to morphologically identify eggs in feces and the difficulty in identifying larvae on pasture (Lichtenfels et al., 2008). The current method of identifying cyathostomin species involves morphological examination of adult stages necessitating the sacrifice of infected horses. Furthermore, species identification of eggs morphologically is not possible and the identification of larvae is difficult and time-consuming using morphological parameters (Gasser et al., 2004).

Research worldwide on the development of diagnostic DNA markers, on the testing of biological and biochemical control agents is hampered by the need to collect specimens from sacrificed horses. However, recent studies (Kaye et al., 1998; McDonnel et al., 2000; Hung et al., 2000; Hodgkinson et al., 2001; Hodgkinson et al., 2005; Lichtenfels et al., 2002; Traversa et al., 2007) have examined molecular

relationships of these species with a view to: (1) preparing a predictive classification and (2) developing molecular markers for use in identification of both pre-parasitic and parasitic stages.

In the present study, we applied a PCR-based RLB hybridization assay for molecular identification of small strongyle species in horses, as efficient tool to monitor the small strongyle populations in horses and to survey emergence of drug-resistant species.

1. MATERIAL AND METHODS

The study was performed in a horse farm (in Central Kentucky) known to be infected with only small strongyles. Horses in this herd had been treated exclusively with ivermectin approximately four times a year since 1990 (Lyons et al., 2008). Occasionally, the foals were given fenbendazole, oxibendazole, and pyrantel pamoate in addition to ivermectin several times before the present study.

Fecal samples were collected from individual horses (n=5). At each collection, the counts of eggs per gram of feces (EPG) were determined using the modified Stoll method (Drudge et al., 1963).

Fecal cultures (Drudge et al., 1963) were done from feces in order to obtained larval and to confirm the absence of *Strongylus* spp. in the herd.

Additionally, four strongyle adults (*Cylicostephanus calicatus, Cylico-stephanus longibursatus. Cylicocyclus nassatus, Cyathostomum catinatum*), morphological identified, were included in the assay as positive controls.

Eggs isolation

Strongyle eggs were isolated from fecal samples with positive fecal egg counts, as described before (Hodgkinson et al., 2005). Harvested eggs were stored in a small volume of water at -20ºC.

DNA extraction

Aliquots of five individual feces samples from horses with positive EPG counts were pooled to obtain three pools which were used for DNA extraction. Genomic DNA was isolated from groups of approximately 6,000-7,000 eggs obtained from each pool. Isolation of DNA was performed according to Hodgkinson et al. (2005) with some modifications. Briefly, a volume of 500 µl of genomic buffer (100 mM Tris - pH 8.0, 50 mM EDTA, 200 mM NaCl, 1% SDS) and 25 µl proteinase K at 20 mg/ml were added to each tube of eggs. The eggs were then incubated at 55˚C overnight. The DNA was extracted with phenol-chloroform, and the DNA precipitated in 2.5 volumes of absolute alcohol.

The DNA was dissolved in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and stored at -20ºC prior to the PCR step.

Genomic DNA was isolated similarly from larvae (two groups of seven larvae each) and the individual adults.

PCR amplification of strongyle DNA and RLB hybridization asay

DNA extracts were used in a PCR assay for the InterGenic Spacer (IGS) of the ribosomal DNA (rDNA) of cyathostomins. PCR reactions were carried out in 25 µl reaction volumes using conserved primers (CY1 forward: 5' GGT CAA GGT GTT GTA TCC AGT AGA G 3' and CY 18Bt -biotinlabeled reverse: 5'/5Bio/ CTT AGA CAT GCA TGG CTT AAT C 3'), which amplify the IGS region from at least 19 different species (Kaye et al., 1998; Hodgkinson et al., 2001, 2003). Amplification reactions contained 1 µl genomic DNA and were performed by the following cycling protocol: 10 min at 94ºC, 35 cycles at 94ºC for 1 min, 60ºC for 1 min, and 72ºC for 2 min, followed by a final extension at 72ºC for 7 min.

From each PCR reaction, 5 µl were subjected to electrophoresis on ethidium bromide-stained 1% agarose gel and visualized under UV transillumination. The IGS amplicons were subjected to the RLB assay using oligonucleotidic diagnostic probes for the most common 13 equine cyathostomin species. Oligonucleotidic probe concentrations and the RLB assay were carried out as in the protocol described by Traversa et al. (2007). In the RLB hybridization, the positive controls were represented by PCR products from the DNA of 4 adults morphologically identified.

Hybridization of PCR products to species-specific probes was revealed by chemiluminescence using SuperSignal substrate (Pierce, Rockford, IL), and images were documented with a FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA).

RESULTS AND DISCUSSION

In the present study, all tested samples were positive for the presence of strongyle eggs. Individual EPG counts from the tested horses varied from 270 to 940. Aliquots of the five individual feces samples from horses with positive EPG counts were pooled to obtain three eggs pools which were used for DNA extraction.

Overall, three egg pools, two pools of larval and four individual adults were successfully amplified by PCR and analyzed by RLB assay (fig. 1).

RLB assay is a relatively new method which is able of simultaneously identifying the most common cyathostomins and to discriminate them from the large strongyles *Strongylus* spp. (Traversa et al., 2007). The method relied on the specific hybridization of *PCR-amplified intergenic spacer DNA fragments of the nuclear ribosomal DNA* to membrane-bound species-specific probes, and enables the non-radioactive hybridization of PCR amplicons with different oligonucleotide probes in a single assay.

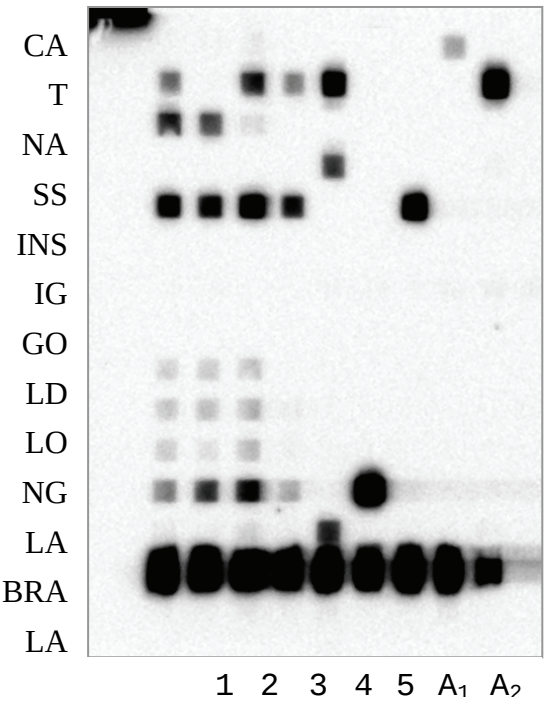

Fig 1. Reverse line blot hybridization assay for the detection and identification of cyathostomin species (abbreviation code for species same as in Table 1) in horses. The oligonucleotidic probes are attached to the membrane in the horizontal direction, and the PCR samples were applied perpendicularly in the vertical direction. The numbered lanes from 1 to 3 represent equine strongyle egg sample-derived PCR products. Lanes 4 and 5 are the equine strongyle larvae sample-derived PCR products. Lanes from A_1 to A_4 are the PCR products from individual cyathostomin adults

The RLB assay revealed in our study the presence of nine species of the 13 most common cyathostomins tested. The results and the proportion of each species identified in this study are presented in the Table 1.

Overall, of the 9 cyathostomin species identified by RLB assay, 7 were detected in the strongyle egg sample-derived PCR products: *Cys. longi-bursatus*, *Cyc. leptostomus*, *Cor. catinatum*, *Cyc. ashorthi*, *Cys. calicatus*, *Cyc. nassatus* and *Cyc. insigne.* The first five species were found in all tested samples, and the last two species were found in 2 of the 3 tested samples.

Five cyathostomin species were detected in the larvae sample-derived PCR products: *Cyc. nassatus*, *Cys. goldi*, Cys. *longibursatus*, *Cys. calicatus* and *Cys. minutus*. All four cyathostomin adults morphologically identified were also confirmed by RLB assay.

No *Strongylus* species were found in the samples analyzed, confirming the absence of these parasites in the herd and the conserved efficacy of ivermectin against large strongyles (Kaplan, 2002).

Table 1

Cyathostomin species identified by the PCR-based Reverse Line Blot hybridization assay using egg, larval and adult parasite-derived DNA

Egg/ larval/ adult	No. of samples used for analysis	No. of samples containing the following strongyle species[a]												
		cat	nass	insig	gold	long	labra	labia	lepto	or	ash	cal	min	Stron. spp.
Egg	3	-	2	2	-	3	-	-	3	3	3	3	-	-
arvae	2	-	2	-	1	1	-	-	-	-	-	1	1	-
Total	**5**	**-**	**4**	**2**	**1**	**4**	**-**	**-**	**3**	**3**	**3**	**4**	**1**	**-**
A_1[b]	1											+		
A_2c	1					+								
A_3[d]	1	+												
A_4[e]	1		+											

cat - *Cyathostomum catinatum*; nass - *Cylicocyclus nassatus*; insig - *Cylicocyclus insigne;* gold - *Cylicostephanus goldi;* long - *Cylicostephanus longibursatus*; labra - *Coronocyclus labratus*; labia - *Coronocyclus labiatus*; lepto - *Cylicocyclus leptostomus*; cor - *Coronocyclus coronatus*; ash - *Cylicocyclus ashworthi*; cal - *Cylicostephanus calicatus*; min - *Cylicostephanus minutus*; Stron. spp. – *Strongylus* spp.

[b]A - *Cylicostephanus calicatus*; [c]A_2 - *Cylicostephanus longibursatus*; [d]A_3 - *Cyathostomum catinatum*; [e]A_4 - *Cylicocyclus nassatus*

The results of this study showed that the small strogyle populations in this herd involved many species, and the magnitude and prevalence of each species population could displays some variations.

Surveys worldwide have reported about 16-24 cyathostomin species in most regions and from 4 to 14 species with a prevalence of 50% or higher (Cavalho et al., 1998; Lyons et al., 1999; Lichtenfels et al., 2001). In a recent study Traversa et al. (2009) studied the anthelmintic susceptibility of the cyathostomin populations in horses from Italy. They identified eight cyathostomin species in pretreatment samples. Also, Cernaska et al. (2009) reported molecular identification by RLB assay of seven benzimidazole-resistant cyathstomin species.

Therefore, our study confirmed that RLB assay is enable the accurate and rapid identification of equine cyathostomins irrespective of their life cycle stage, opening important avenues for a better understanding their biology and epidemiology, and pathogenesis of cyathostomin associated disease.

In particular, this RLB method promises to be a powerful diagnostic tool to determine the role of individual species in the pathogenesis of mixed infections and to elucidate some aspects of cyathostominosis. It could also represent a basic step towards the development of a rapid and simple molecular test for early detection of drug-resistant genotypes of horse strongyle species.

3. CONCLUSIONS

3.1. Genomic DNA, isolated from equine strongyle eggs, larvae and adults was successfully amplified by PCR (for the InterGenic Spacer of the rDNA of cyathostomins) and analyzed by RLB assay.

3.2. Nine cyathostomin species were molecularly identified by RLB assay in the tested samples: Cylicocyclus nassatus, Cylicocyclus insigne, Cylicocyclus leptostomum, Cylicocyclus ashworthi, Cylicostephanus longibursatus, Cylicostephanus goldi, Cylicostephanus calicatus, Cylico-stephanus minutus and Coronocyclus coronatus. All four cyathostomin adults morphologically identified were also confirmed by RLB assay.

3.3. No *Strongylus* species were found in the samples analyzed, confirming the absence of these parasites in the herd and the conserved efficacy of ivermectin against large strongyles.

3.4. The PCR-RLB assay is an useful tool to determine distribution and species-specific occurrence of cyathostomins under field conditions and to discriminate them from the large strongyles, indicating an invaluable way to furthering drug-resistance studies.

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A CLINICAL CASE OF ASYMPTOMATIC PANCYTOPAENIA IN A DOG

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Key Words: pancytopenia, Ehrlichia canis, dog

Summary

A clinical case of asymptomatic pancytopaenia in a 3-year old Cocker Spaniel from Stara Zagora is described. The clinical status of the patient and the results from haematological blood biochemical and serological analyses are reported. On the basis of these examinations, subclinical monocytic ehrlichiosis was detected. The outcome of the applied therapeutic protocol is monitored. The role of the causing agent - *Ehrlichia canis* - is specified with regard to public health.

Pancytopenia is a rarely observed pathological condition in dogs. The causes of its occurrence can be non-infectious contagious – intoxication (thallium), neoplasias (bone marrow), anaemia (immune-mediated aplastic), hepatitis, as well as infectious – ehrlichiosis. Asymptomatic pancytopenia is even less commonly found and described disease.

MATERIALS AND METHODS

The subject of this report is a 3-year-old female dog, 10 kg in weight, named Raya, residing in Stara Zagora. The dog had been regularly immunized for canine distemper, hepatitis, parvovirosis, parainfluenza, leptospirosis, and rabies. The patient was admitted into a private clinic for annual re-immunization.

Blood samples were collected from vena cephalica antebrachii – 5 ml in a vaccutainer with EDTA and another 5 ml in a vaccutainer for blood serum.

The following blood parameters were assayed: WBC x 10^9/l; RBC x 10^{12}/l; HGB, g/l; HCT, %; MCV, fl; MCH, Pg; MCHC, g/l; PLT x 10^9/l; total protein (g/l), albumin (g/l), albumin/globulin ratio (A/G), urea (mmol/l), creatinine (μmol/l), bilirubin (μmol/l), AP (U/l), ALAT (U/l), ASAT (U/l). The absolute neutrophil, lymphocyte, eosinophil, basophil and monocyte counts were calculated. Haematological studies were

performed on an automated counter BC–2800 Vet Auto Hematology Analyzer, Mindray, Korea. Blood biochemical analyses were done with diagnostic kits of Roche Diagnostics GmbH, Germany on a biochemistry analyzer BA–88 Mindrey, Korea.

A serological examination was also done for the following vector-borne infections: monocytic ehrlichiosis, granulocytic anaplasmosis, borreliosis (Lyme disease), and dirofilariosis. A combined ELISA test IDEXX Snap® 4DXTM Test, USA for *Ehrlichia canis* (antibody), *Anaplasma phagocytophilum* (antibody), *Borelia burgdorferi* (antibody), and *Dirofilaria immitis* (antigen) was used.

RESULTS

The information obtained from the anamnesis and the clinical examination of the patient was as followed. The dog had exhibited variable appetite over the course of several months. Sometimes, it was lethargic, yet no permanent behavioral changes were observed. The clinical examination did not detect any systemic aberrations. Only the conjunctivas of both eyes had become pale (Fig. 1). The results from the haematological tests (Table 1) showed a clearly expressed pancytopenia - erythrocytes 1.73×10^{12}/l, leukocytes 1.4×10^{9}/l, and thrombocytes 2×10^{9}/l. All other indicators (except MCH) were also below the normal range. The patient's biochemical indices were within the reference range or slightly increased (Table 1).

Hematology and Biochemistry results	**1st Day**	**7th Day**	**Ref. Val.**
RBC x 10^{12}/l	**1.73**	2.04	5.5 - 8,5
HGB x g/l	42	60	120 - 180
HCT %	13.8	15.6	37 - 55
MCV fl	2	33	60-77
MCH pg -	1.4	1.5	19-23
MCHC g/l	1.73	2.04	320-360
PLT x 10^{9}/l	**2**	60	150-500

WBC x 10^9/l	**1.4**	15.6	6·0 - 17·0
NS x 10^9/l	1.064		3·0 -11·5
LY x 10^9/l	0.112		1·0 - 4·0
EO x 10^9/l	0.168		0·1 - 1·25
MON x 10^9/l	0.056		0·1 - 1·35
BA x 10^9/l	0		0 - 0.1
Total protein (g/l)	77	72	54 -75
Albumin (g/l)	29	31	25 - 45
Globulin (g/l)	48	41	27 - 44
Albumin/Globulin Ratio (g/l)	0,604	0.756	0.60 – 1.1
Urea (mmol/l)	3.45	3.23	3·3 – 8·3
Creatinine (μmol/l)	66.6	79.3	35 - 106
AP(U/l)	91	20	to 108
ALAT (U/l)	42	106	to 55
ASAT (U/l)	59	40	to 25

Table 1. Hematology and biochemistry results in a dog with asymptomatic pancytopaenia
***Ref. Val. = reference values (Kraft and Durr, 1995; Feldman et al., 2000)**

The result from the ELISA examination was positive for *Ehrlichia canis.*

A treatment with doxycycline, 100 mg and prednisolone, 5 mg daily, was initiated.

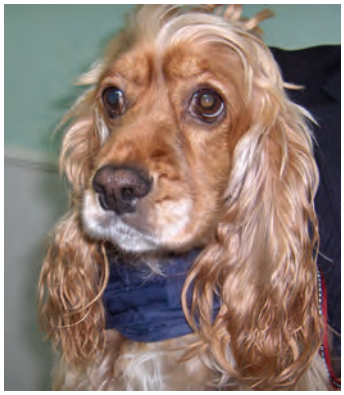

Fig.1. Dog with asymptomatic pancytopaenia

DISCUSSION

The data from the anamnesis, the clinical examination and the haematological/biochemical tests gave reason to assume the occurrence of a vector-borne disease (Harrus et al., 1997). The established aberrations in complete blood counts and especially the pancytopenia are diagnostic finding, which require confirmation or rejection of the vector-transmitted infections encountered in Bulgaria – monocytic ehrlichiosis, granulocytic anaplasmosis, borreliosis (Lyme disease) and dirofilariosis. A simultaneous examination for all these four diseases is necessary because, as it is known, the diseases often occur simultaneously as well (Tsachev, 2009). The result from the ELISA examinations were informative enough to diagnose asymptomatic pancytopenia caused by *Ehrlichia canis*. It is known that the most valuable and informative haematological parameter in clinical patients with ehrlichiosis is thrombocytopenia (Troy et al., 1980; Ristic and Holland, 1993; Waner et al., 1995, Macieira et al., 2005). Thrombocytopenia was also proven in the sub-clinical *E.canis* form (Codner and Farris-Smith, 1986; Waner et al., 1997). During experimental induction, significant leukopenia was evidence in 78% of the dogs with subclinical *E.canis* expression, and in 71% of the cases it was also combined with absolute neutropenia (Waner et al., 1997). The development of severe pancytopenia, a prognostic indicator of a lethal outcome (Shipov et al., 2008), is related to bone marrow damage (Swango et al., 1989; Rikihisa et al., 1994; [b]Harrus et al., 1996). In our case, the results after a 7-day doxycycline/prednisolone therapy showed the desired tendency towards improvement of the haematological indices, and the therapy was continued for 3 more weeks. The decrease in the total blood protein, and the accompanying reduction in globulins was the result of the accurate diagnosis and adequate treatment. The patient exhibited improved appetite and excellent physical condition during both the first week, and after the end of treatment.

It should be emphasized that *Ehrlichia canis* is also a zoonotic agent, detected in people in Venezuela (Perez et al., 1996; Perez et al., 2006). This is why, the treatment of this infection is particularly indispensable.

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HYPERTROPHIC OSTEODYSTROPHY IN FOUR DOGS

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Key words: hypertrophic osteodystrophy, HOD, dog, vitamin C, radiological findings

Summary

The signalment, clinical signs, radiological, laboratory and histopathological findings, aetiopathogenesis and treatment of canine hypertrophic osteodystrophy (HOD) are discussed in the face of four clinical cases with HOD.

Hypertrophic osteodystrophy (HOD) is a developmental skeletal disease in young (2-8 months of age) rapidly growing large and giant breed dogs which was first described as Barlow's disease and subsequently was termed Moeller-Barlow's disease, idiopathic osteodystrophy, canine skeletal scurvy, and metaphyseal osteopathy.

It is manifested with metaphyseal swelling and pain, depression, inappetance, pyrexia, reluctance to stand, and stiff gait (1, 2). A history of recent diarrhea, pneumonia, bronchitis, tonsillitis, or ocular and nasal discharge may precede the onset of lameness (3, 4). Some cases recover spontaneously, but others have multiple relapses and finally necessitate euthanasia because of severe pain, cachexia and debility or other complications.

Abnormalities occur at the metaphyseal regions of the long bones and consist of disrupted and necrotic trabeculae, haemorrhage, haemosiderin deposits and inflammatory cells. This produces characteristic radiographic changes of metaphyses which are usually bilateral. Typically affected bones are radius, ulna, and tibia but other sites of growth such as femur and humerus, metacarpal and metatarsal bones, vertebrae, ribs, and scapula can be similarly but rarely affected (5, 6).

Many causes of the disease such as overfeeding, vitamin C deficiency, excessive dietary vitamin D, excessive dietary minerals and calories, have been speculated till now but the aetiology has not been revealed yet. Recent studies postulated a canine distemper virus as a possible aetiological agent (7, 8).

The aim of the present study was to report the history, signalment, clinical, radiological, haematological, and hystopathological findings in

four dogs with naturally occurring hypertrophic osteodystrophy and to compare these data with other investigations.

MATERIAL AND METHODS

This article describes four cases of hypertrophic osteodystrophy in dogs which have been referred to the Small Animal Clinic of the Faculty of Veterinary Medicine since May 2008 year till May 2009.

All affected dogs appertained to large breeds, between 4 and 6 months of age, three male and one female, and came from the seaside regions: Karnobat - 2 dogs, Bourgas - 1, Sozopol -1. All dogs were not overfed. Vaccines used had been from different manufacturers, all polyvalent including a modified-live distemper virus. Three of four dogs had been previously treated with different kind of medications including calcium supplementation.

All of the dogs underwent clinical and radiological examinations. Radiographs of the affected extremities were performed in two views. Moreover, other radiographs of the axial skeleton were taken.

Haematological examinations and blood chemical analyses have also been done using commercial kits (Human Diagnostica, Germany), automatic haematology analyzer BC-2800 Vet (Mindray, China) and semiautomatic biochemistry analyzer BA-88 (Mindray, China). Urine biochemical and sedimentation analysis were made.

Plasma ascorbic acid concentrations were immediately determined using the sensitive and rapid method of Day et al. (9). In brief, the assay is based on the reduction of ferric chloride by ascorbic acid with the resulting ferrous ion quantified by the addition of 2,4,6-tripyridyl-s-triazine (TPTZ) to form a purple colour with a maximum absorbance at 595 nm. Uric acid interference has been eliminated by the use of a high molarity acetate buffer and by optimizing the amount of TPTZ and ferric chloride used. Protein was removed by addition of 10% trichloroacetic acid.

Bone samples from the first and the third dog’s radial distal metaphyses were obtained using Jamshidi biopsy needle and sent to the laboratory for histopathological examination. Samples were fixed in 10% phosphate-buffered neutral formalin, decalcified, routinely processed, paraffin embedded, and stained with haematoxylin and eosin (H&E).

All animals were treated with azythromycin (Zithromax®, Pfizer) 10 mg/kg b.w. PO daily for 14 days and carprofen (Rimadyl®, Pfizer) 2 mg/kg b.w. PO for 14 days.

RESULTS AND DISCUSSION

Signalment

Breeds at particularly high risk are Great Dane (10), Weimaraner (4), Irish setter, Boxer, German shepherd, Labrador and Golden retrievers (11). Our study confirms partially these risk categories because affected dogs were two German shepherds, one Great Dane, and one Rottweiler (table 1). Investigating breed predilection for developmental orthopaedic disease, Weimaraner was found 21 times more likely to develop HOD compared to mixed breed dogs (12). Similarly, the Great Dane (190x), the Boxer (18.4x), the Irish setter (14.3x), and the German Shepherd (9.5x) were at increased risk for HOD.

HOD typically affects a single puppy in a litter, although there were two accounts of littermates being affected (4, 13). In the Weimaraner HOD is considered a genetic disease, with a heritability of 0.35, and an autosomal recessive mode of inheritance (14).

Clinical signs appear during intensive growth period between 2 and 8 months (11, 15), as was seen in our four cases. Our data were in accordance with the results of Austin et al. (10) and Ozer et al. (15) who reported that male puppies were twice as likely to develop HOD.

Table 1.

Distribution of cases

Case No	Breed	Age	Sex	Vaccination before first crisis	WBC (G/L)	Vit.C (µmol/l)	Ca/P (mmol/l)	AlPh (U/L)
1	German Shepherd	6 months	male	3 weeks	18.8	123	2.94/1.94	164
2	Rottweiler	5 months	male	3 weeks	20.1	58	2.31/1.92	211
3	German Shepherd	4 months	male	1 week	23.1	56	2.50/2.26	151
4	Great Dane	6 months	female	6 weeks	22.0	28	2.78/2.1	183

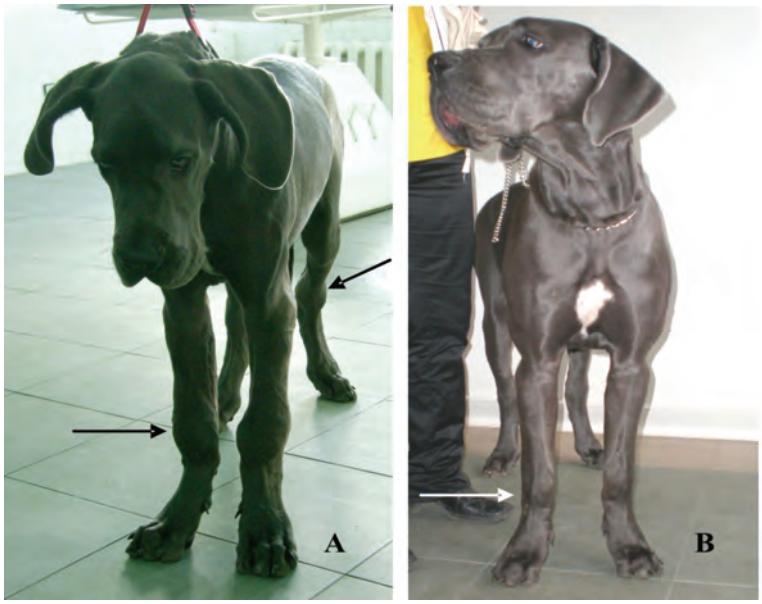


Fig. 1. A Great Dane puppy with the most typical clinical sign of HOD – painful swellings of four legs just above the wrists and hocks (black arrows) at the day of presentation (A) and the obvious clinical improvement three months after initial presentation (B) without any swellings of the limbs (white arrow).

Clinical findings Common clinical signs in our cases were fever (over 40°C), inappetence, depression, reluctance to walk, stiff gait, lameness, firm and painful swellings of the regions above both carpal joints and to a lesser extent above tarsal joints (fig. 1A). These findings had been described by many other researchers (3, 6, 11, 15, 16).

Laboratory findings

No changes were detected in morphological and biochemical blood and urine analyses except for the elevated WBC, blood inorganic phosphate level and alkaline phosphatase (AlPh) activity and increased blood ascorbic acid concentrations (table 1). According to the results of Ozer et al. (15) HOD induced a significant lymphocytosis, increase in serum AlPh, calcium, phosphate, and decrease in total protein and albumin. In other studies (3, 11) haematological and biochemical tests usually showed neutrophilia, monocytosis, and lymphocytopenia, reflecting stress and inflammation. Our data revealed similar changes in blood cells. Elevated alkaline phosphatase activities in all dogs may be explained with the intensive bone metabolism in growing dogs, whereas elevated phosphate levels were probably connected with the preceding calcium supplementation. In spite of previously described

hypovitaminosis C as a causative factor we found normal or slightly elevated plasma vitamin C levels.

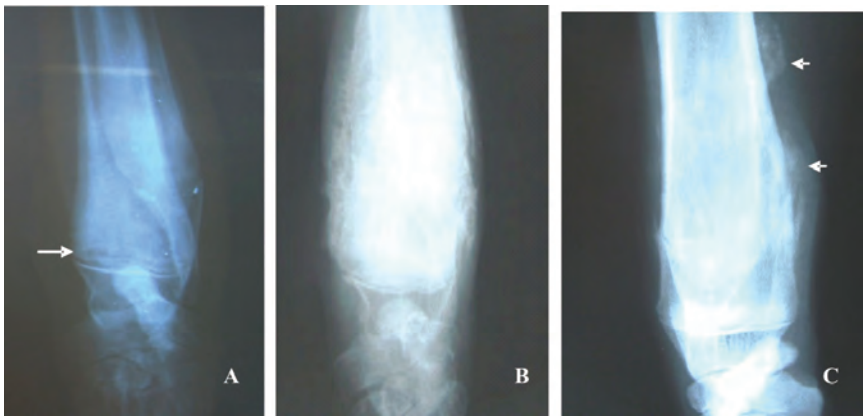


Fig. 2. Radiographs of distal metaphyses of radius and ulna showing a typical appearance (A) of double physeal line– an irregular radiolucent zone just above phiseal growth plate (arrow); later stage of HOD (B) when pseudophysis disappear but osteosclerotic changes with multiple bone cysts and periosteal reaction are visible; almost normal architectonics of metaphyses during inactive healing stage (C) and persistence of new periosteal bone formations (arrowheads).

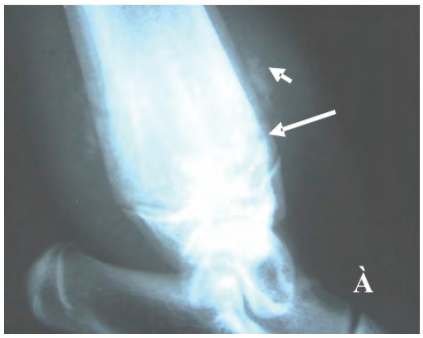
Radiological findings

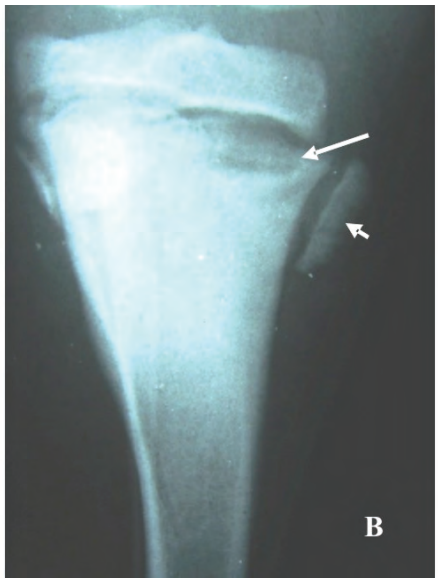
Radiographic findings characteristic for hypertrophic osteodystrophy were: irregular radiolucent line located just above the physeal growth plate of distal radius and ulna, bilaterally (fig.2A), accompanied by periosteal new bone formation as was described previously (3, 6, 11, 15, 16). The same changes were found just under the growth plate of proximal metaphyseal regions of radius, ulna and next to the both physeal growth plates of tibia (fig. 3).

Radiographic findings in our patients were typical and appear gradually in the course of the disease. In the early stage, an irregular radiolucent zone was seen in the metaphysis, separated from the normally appearing growth plate by an opaque band and called pseudophysis or double physeal line (1, 3) that is considered pathognomic for hypertrophic osteodystrophy (16). Later an irregular periosteal new bone formation with metaphyseal enlargement and soft tissue mineralization may be shown.

Once the disease passes into an inactive stage, bone changes undergo a repair and remodeling but some residual diaphyseal distortion and spiculated periosteal exostoses may remain.

Fig. 3. Radiographs of distal (A) and proximal (B) metaphyseal regions of tibia showing a typical radiolucent zone (arrows) next to both physeal growth plates and new periosteal bone formation (arrowhead).





There were some differences in clinical and radiological presentations between the four dogs which were due to different stages of the disease when the animals were presented in the clinic.

Case 1 – There were no typical irregular radiolucent lines over the physeal growth plate but there were osteosclerotic changes at these locations, multiple small bone cysts and new periosteal bone formation (fig. 2B). Abnormal bone proliferations in soft tissues over both great trochanters and in the left kidney were observed (fig. 4).

The disease had had a prolonged history and the animal had been treated with many medications including antibiotics, corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), calcium supplements and vitamins.

The animal was in good health during check-up after 5 months but the bone lesions still persisted.

Case 2 – One month duration of clinical signs with several remissions and relapses instead of treatment using Amoxicillin and Clavulanic acid (Synulox), Doxycycline, intravenous fluids, and calcium supplementation. Soon after weaning, the animal had shown weakness with hind limbs, elevated total white blood cells and blood phosphate level. The radiographic changes were typical.

Case 3 – It was the first clinical presentation of the disease. The animal had not been given any medication before except for calcium supplementation. Typical radiological changes were seen.

Case 4 – X-rays showed typical radiographical changes (fig. 2A). The animal had not been treated before except for calcium supplementation. After diagnosing the animal was cured with Chlornitromycin PO 250 mg TID, chondroprotective agent (Fortiflex), carprofen (Rimadyl), heating up by warm bandages and Hills special formula. Three months after initial presentation there was a remarkable clinical improvement (fig. 1B) without any swellings of the limbs and lameness. X-rays showed an absence of the second physeal cleft, the bones assumed almost normal architectonics, the new periosteal bone formations still persisted (fig. 2C).

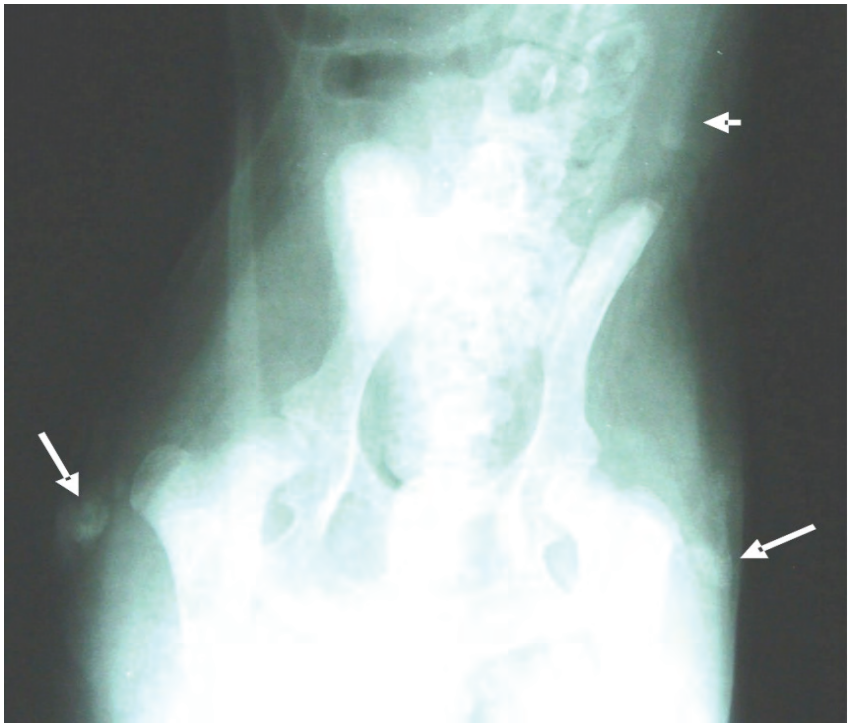


Fig. 4. Ventrodorsal view of pelvis reveals new bone proliferations in soft tissues over the both greater femoral trochanters (arrows) and in the left kidney (arrowhead).

Histopathological findings

Histopathological lesions were characterized by inflammation, necrosis, trabecular micro fractures, and defective bone formation (fig. 5). The inflammation and swelling within fixed location caused damage to the osteocytes, leading to failure of osseous tissue deposition with subsequent weakness of entire bone matrix, culminating in trabecular disruption and fracture (17).

Histological changes were the same as those described by Woodard (13) after necropsy of three Weimaraner dogs died from HOD and by Ozer et al. (15) in their post-mortem hystopathological examination of two dogs died from the disease. Intertrabecular acute inflammation was associated with necrosis, failure to deposit osseous tissue on the calcified-cartilage lattice, and trabecular microfractures. This process led to metaphyseal infraction and separation of the epiphysis. Defective bone formation (osteodystrophy) was considered a secondary process resulting from inflammation of the osteochondral complexes, bone marrow, and periosteum. Enamel hypoplasia also was found to be associated with inflammation of the dental crypt, and abnormal enamel matrix was observed in the developing teeth. We did not found any dental abnormalities in our cases.

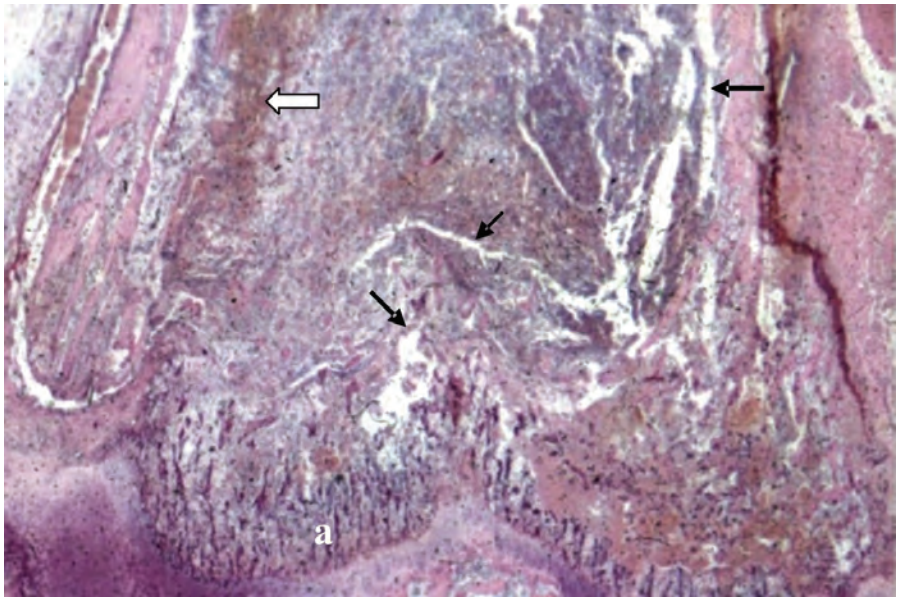


Fig. 5. Hystopathological finding shows trabecular microfractures, necroses (small black arrows), and haemorrhages (white arrow) over intact physeal plate (a) (H.E., 20x).

Etiopathogenesis and treatment

Prior suggested causes of the disease such as overnutrition (18), mineral and vitamins over supplementation, and vitamin C deficiency (19, 20) have been rejected recently. Our results confirmed this statement.

HOD has been linked to excessive calcium content of the diet, because Great Dane puppies fed on a high calcium diet showed HOD and those on a low calcium diet did not show the disease (21). However,

dogs from susceptible lines fed on a restricted calcium diets still showed signs of HOD (5). This suggests that calcium levels influence expression of the inflammatory component of the disease but are not directly causative factor. In our study we did not find neither changes in serum calcium concentration nor abnormal calcium to phosphorus ratios.

HOD was initially compared to skeletal scurvy of children and this led to the hypothesis that the disease reflected a deficiency of vitamin C. However, dogs, unlike humans, can produce the vitamin and do not require supplementation. Moreover, the bone lesion suspected in hypovitaminosis C is osteoporosis, whereas HOD is characterized by excessive bone formation and retarded resorbtion. Our results showed that plasma ascorbic acid levels were within or even over reference range of 18.2-50.7micromol/l (22).

Current thinking has pointed towards an infectious cause of HOD, mainly canine distemper virus (CDV). CDV mRNA has been detected within bone cells of dogs with metaphyseal osteopathy (7) suggesting the role of this virus in the aetiopathogenesis. CDV antigen was found immunocytochemically in haematopoietic marrow cells, osteoclasts, and osteoblasts in metaphyseal bone lesions of young dogs with systemic distemper following experimental and spontaneous infection (23). Instead of lack of clinical signs related to HOD the occurrence of viral antigen in these cells results in defects in bone modelling. Studies of Mee et al. (7, 8) and Mee (24) provide considerable evidence that the defect in osteoblasts (increased number and size), which occur as a primary step in HOD development, occured as a result of increased levels of interleukin-6 (IL-6).

According to Safra et al. (25) and Harrus et al. (26) dogs vaccinated with only trivalent modified-lived canine distemper vaccines have lower risk for HOD compared to dogs, vaccinated with multivalent vaccines. CDV vaccine when administered as a multivalent vaccine could induce the same IL-6 pathway with subsequent inflammation-induced activation of osteoblasts thus leading to HOD in the same manner as virally-induced HOD. In all of our cases animals had been previously vaccinated with multivalent vaccine with modified-live distemper component, so we support the latest statement. The disease developed within six weeks of vaccination in our patients, as was reported by Crumlish et al. (27).

The theory of vaccine-induced HOD may explain the age prevalence of the disease. First HOD incidence (about 8 weeks of age) correlates with loss of maternal antibodies whereas the final age period

of HOD occurring is 6-8 months as long as the puppies still have immature immune system. Dogs less than 6 months of age were identified to be at greatest risk for HOD (10).

Crumlish et al. (27) suggested that MHC alleles at the DLA-DQA1 locus can influence antibody response to vaccination in a group of 33 unrelated Weimaraner dogs but they did not found association between these alleles and HOD. Escherichia coli bacteraemia was detected by Schulz et al. (28) in a dog having had HOD.

Therefore, any pathogen that may activate the cellular pathway of IL-6 induction could lead to osteoblast defects.

There is no specific treatment for HOD, so therapy is aimed at alleviating pain, treating secondary complications, and preventing further injury (5). Most of the investigators used broad spectrum antibiotics, NSAIDs, opioides, and intravenous fluids to treat animals suffering from HOD (6). Lameness disappears and gait improves usually within 5-10 days (16) but multiple relapses may occur. We used azithromycin for treatment of the disease. This macrolide antibiotic was chosen because of its high intracellular level including in phagocyte cells and osteoblasts.

In conclusion, HOD demonstrates a typical radiological sign - double physeal line, but it appears only at early stage of the disease. The relationship between canine distemper virus and metaphyseal osteopathy instead of being obvious remains uncertain and should be investigated further. Hypovitaminosis C theory was not supported by our results. Immunization with only killed or monovalent vaccines may prevent vaccine-induced HOD. Azithromycin is not a specific medication for HOD and the clinical improvement most probably was due to self-limitation of the disease. Animals suffering from HOD have to be supported by painkillers and fluids until clinical signs resolve.

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THE IMPLEMENTATION OF THE STANDARD MICROSCOPIC AGGLUTINATION TEST APPLAIED IN THE SEROLOGICAL DIAGNOSIS OF LEPTOSPIROSIS IN THE NATIONAL REFERENCE LABORATORY

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Key words: leptospirosis, microscopic agglutination, implementation

SUMMARY

The microscopic agglutination test (MAT) is the standard serological test recomanded to be used for the diagnosis of leptospirosis, based on the determination of the antibody level, which react with the antigen reprezented by the different serovars of the *Leptospira spp*.
It is the reference test against which all other serological tests are evaluated.
The study consisted of the assimilation, implementation, optimisation and the *in house* validation of the methodology, regarding the performing of the MAT, based on the recomandations of the OIE Terrestrial Manual, 2008, cap.2.1.9.
The comparative study was carry out with the national omologated and accreditated method, used at present in the serological diagnosis of leptospirosis for animals and people, in Romania.
274 sera samples from different species of animals were examinated and also 15 international reference hiperimmune rabit sera, 23 commercial hyperimmune sera, eight leptospiric hyperimmune sera samples made in IDAH and five sera samples which were sent by Royal Tropical Institute for the International Leptospirosis MAT Proficiency Testing Scheme.
The performance indicators of the method were evaluated by statistical analysis of the results.
The specificity was 100% and the sensitivity was 68% for the herds with leptospirosis suspicion, 93% for the vaccinated animals and 100% for the experimentally infected animals with various serovars of *Leptospira spp.*
The evaluation of the level of antibodies by the standard microscopic agglutination test (MAT), applaied in the serological diagnosis of leptospirosis, based on the recomandations of the OIE Terrestrial Manual, 2008, cap. 2.1.9., is proper for the proposed aim.

In 1918, Martin and Pettit developed the microscopic agglutination test based on the phenomenon of the agglutination and lysis of the *Leptospira* with homologous serum. Since then the method was improved by Schuffner and Mochtar in 1926, Borg-Petersen and Fagroeus in 1949, Wolff, 1954, Carbrez, 1960 and Cole et al., 1973. They tried to standardize factors like the incubation time and incubation temperatures, the density and age of the culture of *Leptospira*, the reading of the samples and the establishment of the end point titre of the antibody (Hartskeerl R.A. et al., 2004).

Antibodies against leptospires are developed in the blood of the animals after 6-8 days from the infection, but the titre is maximum after 3 weeks (1:1600 – 1: 25600), it still maintains for 1-3 weeks, then the antibody level drops off slowly up to 1:50 – 1:100 after many months (Moga, 2002).

As a result of the evaluation for cattle whose main symptomathology were abortion and for a farm without antecedents of abortions, Cordova obtained 100% specificity and 46,5% sensitivity (Cordova et al., 2008).

Antibodies against other bacteria usually do not cross-react with *Leptospira*. However, there is significant serological cross-reactivity between serovars and serogroups of *Leptospira*, although they are usually at a lower level. On the other hand, for the diagnosis of the bovine infected with *Leptospira hardjo*, the sensitivity of the test is about 41%, when a titre of 1/100 is taken as significant and only 67% when the minimum significant titre is reduced to 1/10. Infected animals may abort or be renal/genital carriers with MAT titres below the widely accepted minimum significant titre of 1:100.

Taking in consideration those aspects, the MAT has limitations in the diagnosis of the super-acute and chronic infections in individual animals and in the diagnosis of the endemic infections in herds, requiring the increase of the sensitivity of the test (OIE Terrestrial Manual, 2008).

MATERIAL AND METHOD

MATERIALS:

- The panel of serovars of *Leptospira spp.* whitch of the National Reference Laboratory for Leptospirosis from Romania has been maintaining: *pomona* Pomona 30, *tarassovi* Perepelitsin, *icterohaemorrhagiae* – RGA, *canicola* Hond Utrecht IV, *hebdomadis* Hebdomadis, *wolffi* 3705, *hardjo* Hardjoprajitno, *grippothyphosa* Moscva V, *australis* Ballico, *autumnalis* Akiyami A, *ballum* Mus 127, *bataviae* Swart, *javanica* Veldrat Bataviae 46, *sejroe* M 84, *saxcoebing* Mus 24, *bulgarica* Nicolaevo;
- Reference hyperimmune antisera against serovars of *Leptospira spp*. (Royal Tropical Institute – International WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis): *pomona* Pomona 30, *tarassovi* Perepelitsin, *icterohaemorrhagiae* – RGA, *canicola* Hond Utrecht IV, *hebdomadis* Hebdomadis, *hardjo*

Hardjoprajitno, *grippothyphosa* Moscva V, *australis* Ballico, *autumnalis* Akiyami A, *ballum* Mus 127, *bataviae* Swart, *javanica* Veldrat Bataviae 46, *sejroe* M 84, *saxcoebing* Mus 24, *bulgarica* Nicolaevo;

- 274 sera samples from different species of animals were examinated and also, 15 international reference hyperimmune rabit sera, 23 commercial hyperimmune sera, eight leptospiric hyperimmune sera samples made in IDAH and five sera samples sent by Royal Tropical Institute for the International Leptospirosis MAT Proficiency Testing Scheme.

METHODS:

The standard microscopic agglutination test (MAT – The standard operating procedure SOP 026) - the method which is recomanded by OIE Terrestrial Manual, 2008, cap.2.1.9.;

Microscopic agglutination test (MAT – The standard operating procedure SOP 006) - the national, omologated and accreditated method.

Principle of the test:

The microscopic agglutination test is an antibody-antigen reaction which are read by placing small drops on a microscopic slide under the dark-field microscope.

It is performing serum dilution with antigen, in the microtitration plates with wells, from 1/100 up to 1/102400 or higer to determine the end- point titre.

The microtitration plates are incubated at 29°C ± 1°C, for 2 hours.

The end-point titre is the greatest dilution of serum at which 50% of the leptospires are agglutinated and 50% of leptospires remain free, the density of the free leptospires beeing compared with a control culture which is diluted 1/2 in phosphate bufferd saline.

In order to evaluate the sensitivity, 72 sera samples from suspicious animals were examinated: 57 samples from swine, five from cattle and ten samples from dogs, also, 74 samples taken from vaccinated swine and 12 sera samples from the experimentally infected rabits with various serovars of *Leptospira spp.*

In order to evaluated the specificity, 128 sera samples from animals without antecedents of leptospirosis were examinated: 69 swine, 30 cattle and 29 horses.

2. RESULTS AND DISCUSSIONS

The performance indicators of the method were evaluated by statistical analysis of the results (OIE Terrestrial Manual, 2008, Toacse Gheorghe and Ana Maria Toacse, 2008).

The sensitivity and specificity are the manly performance characteristics of the method which determine together with the disease prevalence in the population, the probability that a given test result reflects the true status of the animal.

The sensitivity (S_n): 100 x serological positive animals / serological positive animals + false-negative animals

For the heard with leptospirosis suspicion (72 samples with 49 positive
results)

$$Sn = \frac{49 \text{ serological positive animals}}{49 \text{ serological positive animals} + 23 \text{ false} - \text{negative animals}} =$$

0,68 x 100 = 68%

For the vaccinated animals (74 samples with 69 positive results)

$$Sn = \frac{69 \text{ serological positive animals}}{69 \text{ serological positive animals} + 5 \text{ false} - \text{negative animals}}$$

$$= 0{,}93 \text{ x } 100 = 93\%$$

For the experimentally infected animals (12 hyperimmune rabit sera)

$$Sn = \frac{12 \text{ serological positive animals}}{12 \text{ serological positive animals}} = 1 \text{ x } 100 = 100\%$$

The specificity (S_p): 100 x serological negative animals / serological negative animals + false-positive animals

128 samples were examined for the evaluation of the specificity

$$Sn = \frac{128 \text{ serological positive animals}}{128 \text{ serological positive animals}}$$

The reproducibility and repeatability

The reproducibility and repeatability are two extreme representations of the precision for each laboratory, a small difference between the reproducibility and repeatability value meaning a robustness test.

Fifteen reference hyperimmnune sera from R.T.I. (Reference Laboratory for Leptospirosis, Amsterdam, Olanda), were examinated.

The reference sera were tested six times for repeatability, in the same day, by the same specialist, who used the same lot of reagents and equipment.

The reference sera were analised for reproducibility six times also, but in the different days, by different specilists, who used different lots of reagents and equipments.

The results were evaluated by the statistical analysis of the end-point titre of the antibodies, which were turn into Log2 and the following performance indicators were calculated:**S_r / S_R:** The standard deviation of the repeatability / reproducibility;

- ✓ **L_r / L_R:** The limit of the repeatability / reproducibility (**L_r / L_R = 2,8 x S_r / S_R** (the difference beetwen two independent mesurements need to be **≤ L_r / L_R**);**CV**: The variation coefficient of the repeatability / reproducibility:

  CV < 10%: small spreading and homogeneous results;
  CV 10 – 30%: middle spreading and little homogeneous results;
  CV > 30%: large spreading and heterogeneous results.

L_r for 15 international reference hyperimmune rabit sera was beetwen 0 and 1,46. The difference beetwen two independent mesurements was **< L_r ,** to agree to the performance criterion of the repeatability.

CV was beetwen 0 and 4,5%, with a small spreading and homogeneous results for all 15 international reference hyperimmune rabit sera.

The statistical results of the repeatability are shown in the table no. 1.

L_R for 15 international reference hyperimmune rabit sera was beetwen 0 and 2,27. The difference beetwen two independent mesurements was **< L_R ,** to agree to the performance criterion of the reproducibility.

CV was beetwen 0 and 5,7%, with a small spreading and homogeneous results for all 15 international reference hyperimmune rabit sera.

The statistical results of the reproducibility are shown in the table no. 2.

As a result of the statistical analysis, it was obtained a small difference between the value of the variation coefficients, which were

determinated in the repeatability and reproducibility conditions, which means a high robustness of the test.

Table no. 1

The repeatability of the reference hyperimmune sera – Royal Tropical Institute

Reference sera	The end-point titre of the antibodies / Log_2						The statistical analysis
Pomona	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	$S_r = 0$ / $L_{r=0}$ C.V. % = 0
Ictero	12800/ 13,64	12800/ 13,64	25600/ 14,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	$S_r = 0,4$/ $L_r = 1,12$ C.V. % = 2,9
Canicola	51200/ 15,64	102400/ 16,64	51200/ 15,64	102400/ 16,64	51200/ 15,64	51200/ 15,64	$S_r = 0,51$ C.V. % = 3,1 $L_r = 1,43$
Hebdomadis	6400/ 12,64	6400/ 12,64	6400/ 12,64	6400/ 12,64	12800/ 13,64	6400/ 12,64	$S_r = 0,4$ C.V. % = 3,1 $Lr = 1,12$
Hardjo	51200/ 15,64	51200/ 15,64	25600/ 14,64	51200/ 15,64	51200/ 15,64	25600/ 14,64	$S_r = 0,52$ C.V. % = 3,4 $L_r = 1,46$
Grippo	51200/ 15,64	102400/ 16,64	51200/ 15,64	51200/ 15,64	102400/ 16,64	51200/ 15,64	$S_r = 0,52$ C.V. % = 3,2 $L_r = 1,46$
Australis	51200/ 15,64	102400/ 16,64	102400/ 16,64	51200/ 15,64	51200/ 15,64	51200/ 15,64	$S_r = 0,52$ C.V. % = 3,2 $L_r = 1,46$
Autumnalis	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	$S_r = 0$ / $L_r = 0$ C.V. % = 0
Bulgarica	3200/ 11,64	3200/ 11,64	3200/ 11,64	3200/ 11,64	3200/ 11,64	3200/ 11,64	$S_r = 0$/ $L_r = 0$ C.V. % = 0
Ballum	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	51200/ 15,64	$S_r = 0,4$ C.V. % = 2,7 $Lr = 1,12$
Bataviae	3200/ 11,64	3200/ 11,64	1600/ 10,64	3200/ 11,64	3200/ 11,64	1600/ 10,64	$S_r = 0,52$ C.V. % = 4,5 $L_r = 1,46$
Sejroe	6400/ 12,64	6400/ 12,64	6400/ 12,64	6400/ 12,64	6400/ 12,64	6400/ 12,64	$S_r = 0$/ $L_r = 0$ C.V. % = 0
Tarassovi	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	$S_r = 0$/ $L_r = 0$ C.V. % = 0

Javanica	12800/ 13,64	12800/ 13,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	S_r = 0,51 C.V. % = 3,6 $Lr_{=}$ 1,43
Saxcoebing	6400/ 12,64	3200/ 11,64	6400/ 12,64	6400/ 12,64	3200/ 11,64	6400/ 12,64	S_r = 0,51 C.V. % = 4,2 $Lr_{=}$ 1,43

Table no. 2

The reproducibility of the reference hyperimmune sera – Royal Tropical Institute

Date of the test	05 .02. 09	2 6.02.	2 0.03.	0 7.04.	1 2.05.	1 3.05	The statistical analysis
Reference sera	The end-point titre of the antibodies / Log_2						
Pomona	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800/ 13,64	S_R = 0/ $L_{R=0}$ C.V. % = 0
Ictero	12800/ 13,64	12800/ 13,64	12800/ 13,64	12800 13,64	12800/ 13,64	25600/ 14,64	S_R = 0,4 C.V. % = 2,9 $L_{R=}$ 1,12
Canicola	51200/ 15,64	102400/ 16,64	51200/ 15,64	102400/ 16,64	51200/ 15,64	51200/ 15,64	S_R = 0,52 C.V. % = 3,2 $L_{R=}$ 1,46
Hebdomadis	6400/ 12,64	6400/ 12,64	6400/ 12,64	6400/ 12,64	12800/ 13,64	12800/ 13,64	S_R = 0,51 C.V. % = 3,9 $L_{R=}$ 1,43
Hardjo	51200/ 15,64	51200/ 15,64	51200/ 15,64	51200/ 15,64	51200/ 15,64	25600/ 14,64	S_R = 0,4 C.V. % = 2,6 $L_{R=}$ 1,12
Grippo	51200/ 15,64	51200/ 15,64	51200/ 15,64	51200/ 15,64	102400/ 16,64	51200/ 15,64	S_R = 0,4 C.V. % = 2,6 $L_{R=}$ 1,12
Australis	51200/ 15,64	51200/ 15,64	10240 0/ 16,64	51200/ 15,64	102400/ 16,64	51200/ 15,64	S_R = 0,51 C.V. % = 3,2 $L_{R=}$ 1,43
Autumnalis	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	12800/ 13,64	S_R = 0,4 C.V. % = 2,8 $L_{R=}$ 1,12
Bulgarica	3200/ 11,64	3200/ 11,64	3200/ 11,64	3200/ 11,64	3200/ 11,64	1600/ 10,64	S_R = 0,4 C.V. % = 3,5 $L_{R=}$ 1,12
Ballum	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	51200/ 15,64	51200/ 15,64	S_R = 0,51 C.V. % = 3,4 $L_{R=}$ 1,43
Bataviae	3200/ 11,64	3200/ 11,64	3200/ 11,64	3200 11,64	3200 11,64	1600/ 10,64	S_R = 0,4 C.V. % = 3,5 $L_{R=}$ 1,12
Sejroe	6400/ 12,64	6400 12,64	6400 12,64	6400/ 12,64	6400 12,64	6400/ 12,64	S_R = 0/ $L_{R=0}$ C.V. % = 0
Tarassovi	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	25600/ 14,64	12800/ 13,64	S_R = 0,4 C.V. % = 2,8

							$L_{R=}$ 1,12
Javanica	12800/ 13,64	12800/ 13,64	25600/ 14,64	25600/ 14,64	51200/ 15,64	12800/ 13,64	S_R = 0,81 C.V. % = 5,7 $L_{R=}$ 2,27
Saxcoebing	6400/ 12,64	6400/ 12,64	6400/ 12,64	6400/ 12,64	3200/ 11,64	6400/ 12,64	S_R = 0,5 C.V. % = 4 $L_{R=}$ 1,4

The comparative results which were obtained by the standard test (SOP 026) and national omologated and accreditated method (SOP 006) are shown in the table nr. 3-6.

Table no. 3

The comparative serological results for the international reference hyperimmune rabit sera from R.T.I. (Reference Laboratory for Leptospirosis, Amsterdam, the Netherlands) and commercial hyperimmune sera from Institute Pasteur and Institute Cantacuzino Romania

The hyperimmune sera	The reference sera R.T.I.		The commercial sera I. PASTEUR		The commercial sera I. CANTACUZINO	
	TITRE/METHOD		TITRE/METHOD		TITRE/METHOD	
	SOP 006	SOP 026	SOP 006	SOP 026	SOP 006	SOP 026
Pomona	12800	12800	400	400		
Ictero	25600	12800			1600	1600
Canicola	51200	51200	12800	12800	100	100
Hebdomadis	12800	12800	6400	6400	800	800
Hardjo	51200	51200	1600	1600	1600	800
Grippo	102400	102400	25600	25600	3200	3200
Australis	102400	102400	25600	25600	102400	102400
Autumnalis	51200	25600	3200	3200		
Bulgarica	3200	3200				
Ballum	51200	51200	1600	3200	1600	1600
Bataviae	3200	3200	3200	3200	3200	6400
Sejroe	6400	6400	200	400		
Tarassovi	51200	25600	6400	3200	1600	1600
Javanica	51200	51200	6400	6400	1600	1600
Saxcoebing	6400	3200	12800	12800		
Wolffi			12800	12800		
The correlation of the titres	11 / 15 = 73%		11 / 13 = 85%		7 / 10 = 70%	

Table no. 4

The comparative serological results for the hyperimmune rabit sera made in N.R.L LEPTOSPIROSIS - I.D.A.H.

The hyperimmune sera	RESULTS		TITRE/METHOD	
	SOP 006	SOP 026	SOP 006	SOP 026
Canicola	POSITIVE	POSITIVE	12800	25600
Canicola	POSITIVE	POSITIVE	1600	1600
Wolffi	POSITIVE	POSITIVE	25600	51200
The hyperimmune sera	RESULTS		TITRE/METHOD	
	SOP 006	SOP 026	SOP 006	SOP 026
Wolffi	POSITIVE	POSITIVE	12800	51200
Grippothyphosa	POSITIVE	POSITIVE	25600	25600
Grippothyphosa	POSITIVE	POSITIVE	12800	25600
Australis	POSITIVE	POSITIVE	51200	51200
Australis	POSITIVE	POSITIVE	51200	51200
The correlation of the titres	8/8 = 100%		4 / 8 = 50%	

Tabel nr.5

The comparative serological results for the sera samples which were sent by Royal Tropical Institute for the International Leptospirosis MAT Proficiency Testing Scheme, 2008

The hyperimmune sera	RESULTS			TITRE/METHOD	
	SOP 006	SOP 026	R.T.I.	SOP 006	SOP 026
Tarassovi	POSITIVE	POSITIVE	POSITIVE	3200	3200
Ictero	POSITIVE	POSITIVE	POSITIVE	6400	3200
Negative	NEGATIVE	NEGATIVE	NEGATIVE	-	-
Australis	POSITIVE	POSITIVE	POSITIVE	25600	25800
Canicola	POSITIVE	POSITIVE	POSITIVE	12800	6400
The correlation of the titres	5 / 5 = 100%			3 / 5 = 60%	

Tabelul nr. 6

The comparative serological results for the sera samples from various animals species with leptospirosis suspicious

SPECIES	Samples	RESULTS	TITRE/METHOD	
		SOP 006 / SOP 026	SOP 026	SOP 006
8 swine	5	POSITIVE	100 (australis)	100(australis)
	1	POSITIVE	400 (australis)	400(australis)
	2	NEGATIVE	-	-
8 swine	1	POSITIVE	100(australis)	100(australis)
	2	POSITIVE	200(australis)	200(australis)
	1	POSITIVE	800(australis)	800(australis)
	4	NEGATIVE	-	-

6 swine	3	POSITIVE	200(australis)	200(australis)
	1	POSITIVE	400(australis)	400(australis)
	2	NEGATIVE	-	-
1 dog	1	POSITIVE	100(australis) 50 (grippo) 50 (sejroe)	100(australis) 50 (grippo) 50 (sejroe)
Species	Sampl es	RESULTS	TITRE/METHOD	
		SOP 006 / SOP 026	SOP 026	SOP 006
4 swine	2	POSITIVE	100(australis)	100(australis)
	2	POSITIVE	200(australis)	200(australis)
6 swine	3	NEGATIVE	-	-
	2	POSITIVE	100(australis)	100(australis)
	1	POSITIVE	400(australis)	400(australis)
3 swine	1	POSITIVE	100(australis)	100(australis)
	1	NEGATIVE	-	-
	1	POSITIVE	200(australis)	200(australis)
2 swine	1	POSITIVE	100(australis)	100(australis)
	2	POSITIVE	200(australis)	200(australis)
1 dog	1	POSITIVE	400(ictero) 400(canicola)	400(ictero) 400(canicola)
1 dog	1	POSITIVE	800(ictero)	800(ictero)
1 dog	1	POSITIVE	800(ictero) 800(canicola)	400(ictero) 400(canicola)
2 dogs	2	POSITIVE	200(ictero) 3200(canicola)	200(ictero) 1600(canicola)
1 dog	1	POSITIVE	100(ictero) 200(canicola)	100(ictero) 200(canicola)
1 dog	1	POSITIVE	200(ictero) 400(canicola)	100(ictero) 200(canicola)
1 dog	1	POSITIVE	800(ictero)	400(ictero)
1 dog	1	POSITIVE	100(ictero)	100(ictero)
4 cattle	1	POSITIVE	400 (bataviae) 200(grippo)	200(bataviae) 200(grippo)
	1	POSITIVE	200(bataviae)	200(bataviae)
	2	NEGATIVE	-	-
1 cattle	1	POSITIVE	100(grippo)	100(grippo)
20 swine	9	NEGATIVE	-	-
	6	POSITIVE	100(australis)	100(australis)
	5	POSITIVE	100(australis) 100(ictero)	100(australis) 100(ictero)
The correlation of the titres	72 samples	64 / 72 = 88,88%		

The values of the end-point titres of antibodies show a correlation beetwen 50 - 88,88% and the difference of the results beetwen these methods was just in one dilution.

3. CONCLUSIONS

3.1. The specificity was 100% and the sensitivity was 68% for the herds with leptospirosis suspicion, 93% for the vaccinated animals and 100% for the experimentally infected animals with various serovars of *Leptospira spp.*

3.2. The values of the variation coefficients for all 15 international reference hyperimmune rabit sera, in the repeatability conditions (< 4,5%) and reproducibility conditions (< 5,7%), means a small spreading and homogeneous results and the method meet the precision criterion. The small difference between the value of the variation coefficients (1,2%), which were determinated in the repeatability and reproducibility conditions, means a high (great) robustness of the test.

3.3. The difference beetwen two independent measurements was less then the limit of repeatability and reproducibility**,** to agree to the performance criterion of the method in the repeatability and reproducibility conditions.

3.4. The comparative results which were obtained by the standard test (SOP 026) and national omologated and accreditated method (SOP 006) showed a correlation of 100% of the positive and negative results.

3.5. The evaluation of the level of antibodies by the standard microscopic agglutination test (MAT), applaied in the serological diagnosis of leptospirosis, based on the recomandations of the OIE Terrestrial Manual, 2008, cap. 2.1.9., is proper for the proposed aim.

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EFFECTS OF EXPERIMENTAL CORTISOL TREATMENT ON THE EVOLUTION OF SOME BLOOD PARAMETERS IN PREGNANT SHEEP

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Key words: cortisol, pregnant sheep, haematological parameters.

Summary

The evolution of the main blood parameters (RBC, WBC, Hb, Ht, PLT, Lym, Gran, albumins, globulins, calcium, phosphorus and magnesium) was searched in advanced pregnant sheep (4.5 months pregnancy age) following a short term (three administrations of two days intervals) of 100 μg/capita of cortisol hemisuccinate treatment versus non-treated pregnant sheep. The results show significant decrease of RBC, WBC and PLT number, immunoglobulin and granulocyte. Total lipids, amylase and fibrinogen levels increased in the same interval of treatment. The other searched parameters, such as calcium, phosphorus, albumins and some globulins remained relatively constant. No influence on clinical physiological status of the sheep was observed.

Hydrocortisone sodium succinate is an ester of cortisol, with inflammatory and decongestiv, antihipotensive, bronchodilator, liver protector and antitoxic effects, intense and relatively fast. Following the intravenous injection, hydrocortisone sodium succinate obvious effects occur after one hour, and the injected dose is eliminated in tens of hours [4, 6]. If human researches on the mechanisms of action and effects of this hormone were the subject of numerous investigation and they are relatively comprehensive known, for the animals, many unknowns persist, according to species and physiological conditions inside of the same species. In this work we do investigated the effects of average duration of this hormone treatment on haematological parameters in advanced pregnant sheep.

1. MATERIAL AND METHODS

To achieve the researches, two groups of clinically normal sheep were established:

- control group (five pregnant sheep of 4.5 months age, of Metis/merino breed;
- experimental group (five pregnant sheep of 4.5 months age of Metis/merino breed.

Animals in experimental group have been given hydrocortisone sodium succinate in dose of 100 μg/cap from two to two days for six days (three administrators). Animals in the control group where given saline solution in the same volume as for the animals from the experimental group, which received hydrocortisone sodium succinate.

The animals were weighted individually to determine the amount quantity of substance to be administered.

Inoculation every two days in a quantity of 100 μg/capita of hydrocortisone sodium succinate aimed to obtain higher blood levels, above physiological limits, exacerbating such a role of this hormone. For blood sampling, only p.v.c. EDTA-type anticoagulant vacutainers were used, tight locked, which were slowly agitated for homogenization.

Sampling was done every two days, respectively 13^{th} (time zero for prior management of the hormone), 16^{th} and 18^{th}, March, 2009.

The tubes were positioned on 45° angle for a fast velocity sedimentation / separation, stored at room temperature, and transported in time for making laboratory determinations, which were done no later than two hours from the moment of sample collection.

Determination of serum hidocortisone was done by an enzymatic method according to Dima Gesellschaft für Diagnostika [1].

Experimental sheep were remained in the herd, which would have been a stress factor, inducing changes of the analysed parameters.

The obtained data were statistically processed and expressed as the mean of each one analyzed group.

2. RESULTS AND DISCUSSION

Administration of cortisol in three reprises, two days between administrations allowed increasing to higher levels of this hormone in plasma, from a value of about 1.48 micrograms per ml to a maximum value of 8 micrograms per ml. Maximum value decreased after the third administration reaching a level of 5.3 micrograms per ml (Fig 1).

The high level of plasma cortisol allowed the recording of its effects on hematology parameters and on the animal health.

On the other hand, a natural increase of cortisol level was foud in control pregnant sheep, following the evolution of the pregnancy process, which correlates with the final stage of gestation [6]. In the short period of the six days of monitoring, the cortisol level in control sheep increased from 2.55 to 3.50 micrograms per ml of plasma.

The results of the exams of blood morphology in the period of the cortisol treatment are presented in table 1. From the data in table 1 results show that some blood morphological parameters were modified by cortisol treatment while the others ones didn't. So, WBC number decreased from 7.6 to $3.62x10^3/mm^3$ following the six days of experimental treatment while in the control, the WBC number remain constant. This decrease seems to be performed probably by granulocytes to whose proportion decreased from 58.8 to 30.0% following the treatment period, while in the control their percent remained constant.

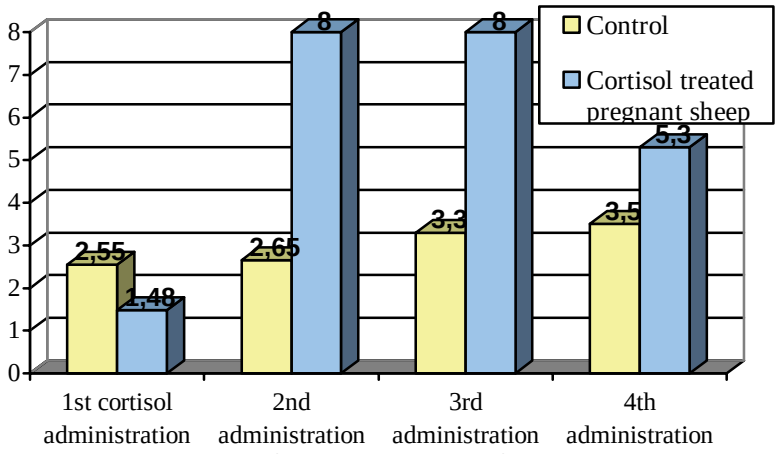

Fig. 1. The evolution of the plasmatic level of the cortisol in pregnant sheep following three administrations of 100 micrograms cortisol three times at two days intervals vs. control sheep

Table 1

The evolution of the blood morphological parameters in cortisol treated pregnant sheep vs. non-treated pregnant sheep following three times cortisol administering for two days interval (mean values from five treated pregnant sheep for each group)

Items	Control pregnant sheep				Cortisol treated pregnant sheep			
	1	2	3	4	1	2	3	4
WBC – 10^3/mm	6.65	6.49	6.53	6.71	7.6	2.11	4.39	3.62
RBC – 10^6/mm	8.01	7.92	8.07	7.98	10.59	6.6	7.1	7.03
HGB – g/dl	8.2	8.5	7.9	8.1	10.7	6.8	7.6	7.3
HT - %	27.4	26.9	27.2	27.4	39.6	21.5	23.1	22.9
PLT – $10^3/mm^3$	164.4	163.7	163.9	164.8	695	153.0	182.5	186.6
MCV – μ^3	43	41	44	42	37	33	33	33
MCH – pg	10.3	9.9	10.8	10.7	10.1	10.2	10.6	10.4
MCHC – %	30.1	29.8	29.3	30.4	27	31.4	32.7	32
Lym %	69.2	64.7	65.5	63.3	33.3	64.4	66.6	58.2
Mo %	4.2	4.4	4.1	4.3	3.9	8.8	6.1	9
Gran %	26.6	26.8	26.5	26.3	58.8	29.8	27.3	30.8

The biochemical parameters of blood plasma stands an increase of fibrinogen from 196 to 355 mg/dl compared with control group of sheep that plasma fibrinogen level remained practically constant (Table 2). Also, plasma total lipids increased in the group treated with cortisol from 172 mg/dL to 270 mg/dl as a massive mobilization of these energy storage to respond to the requirements of the organisms to a possible stress. Blood glucose concentration remained relatively constant even it is well known that cortisol is generalely hyperglycaemic by priming gluconeogenic mechanisms [6]. Note increased activity in serum amylase which presented values significantly higher in cortisol-treated group compared with controls: from 20 to 34 U / L, compared with 3-5 U / L in controls.

The other analyzed biochemical parameters (such as cholesterol, urea, creatinine) showed nosignificant change during the experimental treatment with cortisol in pregnant ewes compared with untreated witness.

Table 2

The evolution of the plasmatic biochemical parameters in cortisol treated pregnant sheep vs. non-treated pregnant sheep following three times cortisol administering for two days interval

Item	Control pregnant sheep				Cortisol treated pregnant sheep			
	1	2	3	4	1	2	3	4
Blood glucose concentration – mg/dl	68	65	67	64	59	61	60	64
Total lipids – mg/dl	231	223	245	219	172	203	294	270
Amylase – U/mL	3	5	4	3	21	33	26	34
Triglycerides – mg/dl	33	35	29	32	24	25	10	15
Cholesterol – mg/dl	79	77	73	74	59	70	73	60
Urea – mg/dl	40	38	41	40	34	34	33	38
Creatinin – mg/dl	0.9	0.83	0.86	0.91	0.78	0.71	0.78	0.76
Fibrinogen – mg/dl	395	391	402	397	195	313	163	355

No modification was noted on calcium, phosphorus, Ca/P ratio and iron levels along to the cortisol treatment in pregnant sheep (table 3).

Table 3

The evolution of some plasmatic biochemical inorganic elements in cortisol treated pregnant sheep vs. non-treated pregnant sheep following three times cortisol administering for two days interval

Item	Control pregnant sheep				Cortisol treated pregnant sheep			
	1	2	3	4	1	2	3	4
Total calcium – mg/dl	9.2	9.1	9	9.2	8	8.8	8.4	8.2
Ionic calcium – mg/dl	3.92	3.80	3.76	3.94	3.48	3.58	3.38	3.48
Phosphorus – mg/dl	8.05	8.21	7.94	8.13	4.33	5.17	5.09	4.86
Iron –μg/dl	121	113	117	124	100	130	81	66

Concerning the albumin level evolution, this blood parameter remained relatively constant despite of the well-known proteolysis effect of the cortisol in most mammals: 41.01 – 44.67% from total plasmatic protein level (table 4). Gamma globulins of treated pregnant sheep group, in exchange, presented a lower percent compared to control group: from 32.29 to 9.14% in experimental group compared to about 29% in control group.

Corticosteroids inhibit pro-inflammatory cytokine production, including that of interleukin-1. Even in very low concentrations they inhibit the synthesis of a variety of pro-inflammatory enzymes, including the macrophage products collagenase, elastase and plasminogen activator

Large intravenous doses of corticosteroids given to normal human volunteers increase the numbers of circulating neutrophils but decrease peripheral lymphocytes, eosinophils, and monocytes.

Table 4

The evolution of electrophoretic plasmatic protein levels in cortisol treated pregnant sheep vs. non-treated pregnant sheep

Items	Control pregnant sheep				Cortisol treated pregnant sheep			
	1	2	3	4	1	2	3	4
Albumins - %	56.27	57.02	57.13	56.87	44.01	42.15	39.39	41.67
Alpha 1 globulins- %	2.2	2.1	2.4	2.3	1.59	1.55	2.51	1.73
Alpha 2 globulins - %	2.98	3.01	2.93	2.97	3.13	3.52	4.45	4.73
Beta - globulins %	8.61	8.69	8.58	8.63	8.94	10.93	11.62	12.73
Gamma - globulins %	29.94	29.56	29.37	29.79	32.29	31.84	22.03	19.14

A decrease of absolute synthesis of albumin, no change in that of fibrinogen and an increased synthesis of transferrin were observed 3h after intraperitoneal administration of a pharmacological dose of 5 mg of cortisol [5].

Studies of different authors revealed that stress and prolonged or excessive administration of glucocorticoids in therapeutic purposes make inhibition on the marrow and on lymphatic tissue [2,4,7].

3. CONCLUSIONS

3.1. Pregnancy advanced period is characterized by a natural slowly increase of blood cortisol concentration, perhaps linked with near delivery time;

3.2. Using of experimental hydrocortisone sodium succinate to pregnant sheep determined some changes dominated by installing of blood leukopenia, decrease of platelets, gamma-globulins and blood red cells number and increase of lipids and fibrinogen.

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BEHAVIOR INTERFERENCES IN SHEEP, ACCORDING TO PHYSIOLOGICAL STATUS, ONTOGENETIC DEVELOPMENT AND SOCIAL ORGANIZATION-A REVIEW

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Key words*:* behavior, sheep, age, social hierarchy

In many countries, sheep has a millenary tradition. Sheep (Ovis Aries) can be increased and operated without major investments in different areas.
After the compared research of the instincts - researchers turned their attention primarily on the behavior, genetically imprinted. In sheep, the social hierarchy is established in a relatively short time, they having well defined positions within the group. Sheep are known to herd instinct, because they are timid and frightened animals. This instinct varies according to race.
Generally, the sheep form stable social groups and social organization influence the type of grazing herd. Age studies conducted on the aggressive behavior of sheep, indicates that older animals are more involved in fighting.

In many countries raising sheep is a millenary tradition. The diverse products of sheep, the low consume of energy, the reduced demands on the nature of feed they consume, gives raising and exploiting these species the basic character of an activity and future sustainable.

Sheep (*Ovis aries*) can be raised and exploited without major investments in different arias of climate, vegetation and geographic forms. From sheep to obtain products, particularly applied internally and externally, especially given that, as EU member, Romania has no quota limit for any of the productions derived from them. So, thanks to their biological characteristics related with obtaining food (meat, milk), as well as materials for the light industry (wool, fell), sheep are a very important asset for domestic economy, something that makes them a highlight in scientific research.

The main problem is the ethological research of the relationship between innate and acquired behavior in sheep. By comparing instincts – etiologists turned their attention first of all on the imprinted behavior of sheep [3,5]. Lorenz, referring to the learning individual process, sustained the idea that an animal can learn based on his heritage and that this predisposition it is not equally developed in different species.

Each animal community has groups of individuals that include adults as well as youngsters and new born. Between individuals of the

same group or different groups certain reports are established, that are most of the time peaceful [9].

In sheep, the social hierarchy is established in a relatively short time, they having clearly defined positions within the group.

Hierarchy once established ensures harmony enjoyed by the whole group and individuals, even if leaders have priority access to food, water and resting place [6].

The group leader has always a straight forward position, raised head and it moves without restrictions of space. On the other hand, the subordinate animals prefer quiet places, carefully watching their leader. Their access to food is discontinuous, but even then their attitude shows prudence because they are always alert and prepared to leave in a hurry in case of attack from dominant animals [10].

Sheep are well known for their herd instinct. Because they are scary animals, they will group (in herd) in order to feel protected.

This is the only protection they have against predators, because it is harder for a predator to hunt a sheep in a flock, only one lost from some authors' point of view, the herd instinct varies in each breed [15]. So, even since birth, mothers encourage their lambs to follow them around, later being learned to follow old members of the herd [8].

Usually, animals are led by dominant members of the herd, followed by the rest of the submissive herd. If the herd has a ram, usually he leads [12]. According to some studies, sheep have to have a five individual group to determine a normal herd behavior [10].

Sheep are social beings; so, when they are grazing they have to make visual contact to each other, making handling them so much easier.

Seeing is a very important part of communication that is why when grazing, sheep maintain visual contact to each other [9,14]. Each sheep raises its head to see the other sheep position, confirming the herd instinct at this specie. So seeing another sheep reduces stress [15].

Sheep have panoramic view of 330° - 360° and two-dimensional of 25°-50°. It is thought that they can see in colors and can distinguish: black, red, yellow and white [9]. Following an experimental study made on sheep, it was established that, each sheep kept under observation another two sheep under 1100 angle. This eye contact assures animal orientation and gives them a sense of security [12].

Sheep becomes nervous if separated from the herd [7]. Besides the fact that the herd instinct serves as a protection mechanism against predators, this helps people to handle big herds of sheep. This instinct

makes sheep easier to handle or move with the help of a guard dog [4, 11].

Domestication and the contact with people strengthened the herd behavior. Domestication favors sheep docile behavior [1]. The herd instinct is not always a good idea for the animals from this specie, because when a sheep moves the others will follow.

Science literature, quoted a case in witch herd instinct was so strong that caused the death of 400 sheep, in East Turkey (2006). They died after one of them tried to cross a 15 meter deep gap, and so the others followed it [15].

The domination-dependency relationships between the herds have been analyzed by Squires and Daws in 1975 (quoted by [2]), they have found a linear hierarchy at Merinos and a less rigid structure at Border Leicester when competitive feeding begins.

Usually, sheep form stable social groups and, so, the social organization of the herd influences grazing types because animals are not randomly dispersed in the environment but there may be groups, who choose to care resources and vegetation features [13].

Young lambs formed close links between them and become dependent on each other beings (dogs, people).

The number of dominant fights between herds is bigger in the same age groups or same sex than in joint groups (Stolba, 1990 quoted by [15]). Apparently, sheep social system formed after using antipredator and feeding strategies, based on learned traditions (Festa-Bianchet, 1991, quoted by [15]).

Age studies made on sheep aggressive behavior indicate that elderly animals are more into fighting (Stolba, 1990, quoted by [15]). Family groups have a less fighting and affection tendency, so a social stable herd is less affected by heterogeneous environment than the groups that are not socially integrated [4,6].

Some studies have also shown that different types of breeds have different structures (Arnold, 1981, quoted by [15]):

- Merinos: is a well unified and tight herd that rarely forms subgroups. Sheep graze near one another and disperse only when food is less;
- Australian Southdown, usually, form subgroups and associate when grazing but not when they are resting;
- Other breeds form more subgroups.

In subgroups, the herd has a certain social identity, because the members change constantly. An important aspect regardless the breed is that herds coming from other sources do not integrate well into a social

homogenous group. That means that, if the cart is big enough, each group will use a different area even if the better food is found only in one spot [3, 9].

In a gregarious breed, such Merinos, the herd moves as a one body and can not graze on pastures that are not uniform in terms of food abundance. The net effect of this behavior, to a large scale, is that of increasing or decreasing the productivity rate [14].

Certain sheep breeds have well defined resting customs, named camping. Choosing camping spots is important because this custom is different between camping during the day and camping during the night [10].

Sheep know very well the road between the water source and the camping spot (Squires, 1981, quoted by [15]). Merinos camp on high grounds when the weather is cold, near water and in shadow when the weather is hot.

So, in summer, if there are bowers on the pasture, sheep will protect them selves against sun, if there are not bowers they gather around in groups covering their heads (Schreffer and Hohenboken, 1980, quoted by [14]).

On a cold weather using artificial wind defenders supplies protection against wind and reduces the mortality rate. So, according to some studies lamb mortality falls from 35,5%, in those not sheltered, to 8,8% in those sheltered, especially when the temperature is lower than 5°C in the first 6 hours after birth [12,4]. Choosing shelters is important, that is why the behavior and natural environment preferred by sheep is considered.

So, trough rigorous selection and technology breeding adaptations and exploiting sheep, synthetic lines were obtained, with a big productive capacity, according to nowadays demands.

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VALIDATION OF PCR-BASED METHODS FOR GMO IDENTIFICATION AND QUANTIFICATION

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Key words: genetically modified organism (GMO), PCR, performance requirements

SUMMARY

Validation is the process establishing the suitability of an analytical method for a particular purpose. Various guidelines defining procedures for validation of molecular methods have been developed. However, there is no universally accepted practice for assay validation, and often, subjectivity plays an important role in the interpretation of validation studies' results. The key to rational validation studies relies upon the harmonization of procedures for their design and interpretation of results.
The recommendations on how methods for genetically modified organism (GMO) analysis shall be evaluated and validated by the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) in the context of Commission Regulation (EC) No. 1829/2003 can be helpful in such respect. The scope of the validation studies outlined in this paper was to evidence that GMO testing methods used in our laboratory meet the acceptance criteria described in the ENGL document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (13 October 2008).

The solution chosen in our laboratory for the evaluation of analytical procedure performances was to conduct 3 separate validation studies: (*1*) validation of the DNA extraction method for a specific matrix, such as soybean flour and determination of the resulting DNA quality (length and structural integrity), quantity, and purity (e.g., absence of PCR inhibitors); (*2*) validation of the PCR method for the species-specific sequence; and (*3*) validation of the PCR method for the GM-specific sequence.

Validation of DNA extraction method consists of assessing the ability of the extraction methods to produce pure DNA extracts of acceptable quality and sufficient quantity to allow it to be used in qualitative and/or quantitative PCR analyses. Given such a DNA solution, methods for species-specific and GM-specific PCR targets could be validated separately (Holst-Jensen et al.,2004).

The proposed ENGL performance criteria for PCR-based methods are:

Applicability – the description of analytes, matrices and concentrations to wich the method can be applied;

Specificity – property of a method to respond exclusively to the characteristic or analyte of interest;

Dynamic Range – the range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision;

Trueness – the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value;

Amplification Efficiency – the rate of amplification that leads to a theoretical slope of -3,32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $10^{(-1/slope)} - 1$;

R^2 Coefficient – the correlation coefficient of a standard curve obtained by linear regression analysis;

Precision ● Relative Repeatability Standard Deviation (RSD_r) – the relative standard deviation of test results obtained under repeatability conditions;

● Relative Reproducibility Standard Deviation (RSD_R) - the relative standard deviation of test results obtained under reproducibility conditions;

Limit of Quantification (LOQ) – the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Limit of Detection (LOD) - the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single-laboratory validation;

Robustness – a measure of the method's capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing).

GM soybean GTS 40-3-2 (RoundupReady) identification and quantification protocols are the testing methods for which the assessment of performance requirements were described.

MATERIAL AND METHOD

The process of analysing for presence of GM soybean GTS 40-3-2 consists of several steps: sampling of the material to be analysed, homogenization of the sample, isolation/purification of DNA, setting up two PCRs, one for the GMO (RoundupReady soybean) and one for the

species of interest (soybean) and quantification to determine how much genetically modified material the sample contains (Berdal et al.).

For DNA isolation, a commercially available kit GeneSpin (Eurofins, GeneScan) was used in accordance with the manufacturers' instructions, starting from 200 mg homogenized sample.

DNA concentration was determined by measuring the absorbance of the DNA solutions at 260 nm using an Eppendorf BioPhotometer Plus. DNA integrity was checked by loading 15 µL of DNA extract on a 0,8% agarose gel stained by ethidium bromide, at a constant amperage (60 mA), 90 min.

The GMO*Quant* RoundupReady Soy DNA Quantification Kit (LC) (Eurofins, GeneScan) indicated the presence of DNA from Roundup Ready soy by detecting a DNA fragment of the 35S-promoter sequence of the cauliflower mosaic virus, and the chloroplast transit signal sequence of *Petunia hybrida*; the endogenous reference target sequence lectin served as both a control for DNA integrity and as a reference for relative quantification.

Determination of the copy number by real-time PCR methods involved the establishment of calibration curves based on analysis of a set of calibrators such as plasmid DNA (pDNA).

RESULTS AND DISCUSSION

The aim of a DNA extraction procedure is to provide DNA of suitable quality for subsequent analysis, so in order to validate a DNA extraction method for a specific matrix we had to determine the resulting DNA quality, quantity, and purity (Thompson et al.,2002).

Six soybean flour test portions were processed on 3 different days. The suitability of the extracted DNA for detection and quantification experiments was verified by UV-spectroscopy (yield, purity) and genomic DNA electrophoresis (quality).

DNA concentration was measured as amount of the analytes/volume of solution and compared to the working concentration described in the PCR protocol (40 ng/µl). The concentration values obtained for all the DNA extracts were higher than 400 ng/µl, demonstrating the efficiency of the extraction method.

DNA fragmentation state

The molecular weight of the extracted DNA samples were determined by agarose gel electrophoresis. The distinct fluorescent bands of high molecular weights (>20000 bp) demonstrated that

breakage of genomic DNA into smaller DNA fragments was limited for all the DNA extracts.

Purity of DNA extracts

The absence of PCR inhibitory compounds in the DNA samples was demonstrated by Real-time PCR for the detection of endogenous control gene lectin, on serial dilutions of the DNA extracts (DNA solutions adjusted to a concentration of 40 ng/µl – "undiluted" samples).

The Ct values of the four diluted samples were plotted against the logarithm of the dilution and the Ct value for the „undiluted" sample (40 ng/µl) was extrapolated from the equation calculated by linear regression. Subsequently, the value of the measured Ct was substracted from the extrapolated Ct for the „undiluted" sample (ΔCt).

Tabel 1

Comparison of extrapolated Ct values versus measured Ct values for eighteen DNA samples extracted from soybean flour (amplification of soybean lectin gene)

DNA extraction	Slope (a)	R^2	Ct extrapolated (b)	Ct measured	ΔCt^*
1.1	-3,3	0,9935	23,85	23,46	0,39
1.2	-3,3	0,9929	23,735	23,67	0,085
1.3	-3,3	0,9995	23,27	22,96	0,31
1.4	-3,6	0,9997	22,915	22,88	0,035
1.5	-3,79	0,9997	22,915	23,00	0,085
1.6	-3,3	0,9984	23,86	23,54	0,32
2.1	-3,2	0,996	23,25	23,71	0,46
2.2	-3,4	0,9997	23,61	23,29	0,32
2.3	-3,2	0,996	23,265	23,52	0,255
2.4	-3,3	0,9991	23,685	23,63	0,055
2.5	-3,4	0,9997	22,97	23,00	0,03
2.6	-3,6	0,9996	22,695	22,85	0,155
3.1	-3,4	0,9962	23,25	22,96	0,29
3.2	-3,3	0,9994	23,775	23,54	0,235
3.3	-3,2	0,9987	23,13	23,03	0,1
3.4	-2,98	0,9882	23,08	22,99	0,09
3.5	-3,2	0,9994	23,815	23,55	0,265
3.6	-3,4	0,97	23,82	24,04	0,22

* ΔCt = | Ct extrapolated - Ct measured|

All ΔCt values presented in table 1 were < 0,5, demonstrating that PCR inhibitors are absent in the extracted DNA solutions.

The assessment of the performance criteria for PCR-based methods comprised the assays described below.

Applicability

For both PCR methods, serial dilutions of DNA isolated from three different groups of matrices (raw materials, end products, feeds) were tested.

Tabel 2

Comparison of extrapolated Ct values versus measured Ct values for DNA samples extracted from different matrices (amplification of GM-specific sequence and amplification of soybean lectin gene)

	Undiluted 200 g/5µl	Serial dilutions			Slope (a)	R^2	Ct extrapolated (b)	ΔCt^*
DNA extraction	1:1 Ct measured	1:4	1:16	1:64				
Raw material 1RRS	31,59	33,33	35,45	37,48	-3,5	0,99	31,27	0,32
Raw material 1L	26,05	27,71	29,53	31,48	-3,1	0,99	25,803	0,247
Raw material 2RRS	31,66	33,39	35,43	37,26	-3,2	0,99	31,49	0,17
Raw material 2 L	25,68	27,43	29,56	31,50	-3,4	0,99	25,427	0,253
Feed 1RRS	28,69	30,76	32,79	35,34	-3,8	0,99	28,383	0,307
Feed 1L	27,51	29,60	31,76	33,77	-3,5	0,99	27,54	0,03
Feed 2 RRS	29,69	31,90	34,20	36,32	-3,6	0,99	29,72	0,03
Feed 2 L	28,54	30,69	32,71	35,06	-3,6	0,99	28,45	0,09
	Undiluted	Serial dilutions			Slope (a)	R^2	Ct extrapolated (b)	ΔCt^*
DNA extraction	1:1 Ct measured	1:2	1:4	1:8				
End product/1 L	30,55	31,91	32,90	33,71	-3,0	0,99	31,04	0,49
End product/2 L	30,33	31,82	32,90	34,13	-3,8	0,99	30,64	0,31

R^2 of linear regression is > 0,99 for all DNA samples and the slope values are between -3,1 and -3,6, with three exceptions (amplification of GM-specific sequence for one of the feed samples -3,8; amplification of lectin gene for the end products - 3,0 and -3,8).

The values of the ΔCt (<0,49) for all the different tested matrices demonstrated the applicability of both PCR methods and the absence of interference effects with other analytes.

Specificity

Specificity of the PCR for the GM-specific sequences was showed by demonstrating that the method doesn't produce amplification signals for non-target transgenic events or non-transgenic material (GA21, Bt176, Bt11, MON810, T25, StarLink, wild type soybean lines).

Specificity of the PCR for the species-specific sequence was showed by demonstrating that the method doesn't produce amplification signals for the closely related species.

Dynamic Range

For both PCR systems, we tried to establish the lowest and the highest concentrations that maintain a linear relationship between the concentration and the response of the methods. These concentrations are presented in table 3.

Tabel 3

Measurement limits of PCR systems (lectin and GM-specific sequence) "GMOQuant RoundupReady Soy (LC)" – Eurofins, GeneScan

PCR system	Minimum quantification limit	1/10 x target conc.	5 x target conc.	Maximum measurement limit
RRS	10 copies/5 µl	<50/5 µl	<2500/5 µl	<10240 copii/5 µl
Lectin	10 copies/5 µl	<50/5 µl	<2500/5 µl	<81920 copii/5 µl
Absolut quantification	0,08%	<0,09%	<4,5%	<100%

The limits presented in table 3 showed that the dynamic range of the methods include the 1/10 and at least 5 times the target concentration (target concentration = threshold relevant for legislative requirements). The range of the standard curves for both real-time PCR systems allows testing of samples throughout the interval comprising 0,09% and 4,5% for a 0,9% GMO concentration or 50 and 2500 genome copies if the target is 500 copies.

Precision – Repeatability, Reproducibility

Repeatability expresses the precision under the same operating conditions over a short interval of time. Reproducibility expresses within-laboratory variations: different days, different analysts, different equipment, etc.

RSD_r (%) of 15 test results obtained by quantifying ERM-BF410d under repeatability conditions was < 25% (9,44%, with the average value of 0,84%). RSD_R (%) of 30 test results obtained by quantifying ERM-BF410d and a sample containing 0,08% GM DNA, under reproducibility conditions, by two analysts on two different days was <

35% for a concentration >0,2% (16,02%, with the average value of 0,91%) and <50% for a concentration <0,2% (31,32%, with the average value of 0,086%).

Trueness

How well a method performs must be tested through comparison with samples of known content. The set of six certified reference materials with different mass fractions of genetically modified (Roundup Ready) soya beans (ERM-BF410), produced by IRMM were quantified in order to determine the closeness of agreement between the average value obtained from a series of test results and an accepted reference value.

The obtained values for all six CRMs were within ± 25% of the accepted reference value (table 4).

Tabel 4

Comparison of the average values obtained for CRMs with the accepted reference values

CRM	Accepted reference value – 25%	Obtained value	Accepted reference value + 25%
ERM-BF410a	**< 0,03%**	NA	-
ERM-BF410b	**0,075 %** (0,1%-0,025%)	0,1%	**0,125** (0,1%+0,025%)
ERM-BF410c	**0,375%** (0,5%-0,125%)	0,49%	**0,625** (0,5%+0,125%)
ERM-BF410d	**0,75%** (1%-0,25%)	0,92%	**1,25** (1%+0,25%)
ERM-BF410e	**1,5%** (2%-0,5%)	1,97%	**2,5** (2%+0,5%)
ERM-BF410f	**3,75%** (5%-1,25%)	5,43%	**6,25** (5%+1,25%)

Amplification Efficiency, R^2 Coefficient

Validation of the quantification experiments requires the verification of the range of values obtained for the slope and the correlation coefficient of the standard curve ($-3,1 \geq$ slope $\geq -3,6$ and $R^2 \geq 0,98$).

Limit of Quantification (LOQ)

160 mg ERM-BF410b were homogenized with 40 mg ERM-BF410a resulting in a sample of 200 mg with 0,08% GM content. The sample was processed for DNA isolation, the resulting concentration adjusted to the working concentration described in the PCR protocol (40

ng/µl) and subsequently quantified. The average value of 15 test results obtained under repeatability conditions was 0,08%, with RSD_r 21,68%.

This assay demonstrated that, for a 0,9% nominal value, LOQ is less than 1/10 of the value of the target concentration (0,09%), with an $RSD_r \leq 25\%$.

Limit of Detection (LOD)

80 mg ERM-BF410b were homogenized with 120 mg ERM-BF410a resulting in a sample of 200 mg with 0,04% GM content. The sample was processed for DNA isolation, the resulting concentration was adjusted to the working concentration described in the PCR protocol (40 ng/µl). A number of 20 PCR replicates were tested. Amplification signals were produced for all 20 replicates.

This assay demonstrated that, for a 0,9% nominal value, LOD is less than 1/20 of the value of the target concentration (0,045%), the presence of the analyte being detected in at least 95% of the replicates.

Robustness

For both PCR systems, the method capacity to remain unaffected by deliberate deviations was measured. The results obtained for ERM-BF 410 CRM_s after utilization of different thermal cyclers, modification of the temperature profile (annealing temperature) or modification of the number of cycles in the PCR profile did not deviate more than ± 30%.

3. CONCLUSIONS

3.1. A rigorous evaluation of methods performance taking into account all performance indices simultaneously allows a correct discrimination among alternative methods.

3.2. The final analytical result is only valid if valid modules are used throughout the analytical procedure; the modular validation approach increases the probability for a successful validation.

3.3. Beside validation requirements, GMO analysis laboratories should participate in proficiency tests organised by independent bodies, to regularly test and demonstrate that their analysis are reliable and accurate.

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THE INFLUENCE OF TEMPERATURE AND GAS MIXTURES ON GROWTH AND SURVIVAL OF *CAMPYLOBACTER JEJUNI* IN CHICKEN MEAT

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Key words: *Campylobacter*, microaerobic, temperature, growth.

SUMMARY

Campylobacter germs are a common cause of food-borne outbreaks in the EU; in 2007, campylobacteriosis was the most frequently reported zoonotic disease in humans.
The growth and the survival of *C. jejuni* were studied using various thermal schemes and different gas mixtures. The samples were incubated at 37°C, 20°C and 4°C, in vacuum atmosphere, aerobic conditions and optimal conditions for the growth of *Campylobacter spp*.
At 37°C, the cell counts of *C. jejuni* strain increased regardless of atmospheric composition, while at 20°C and 4°C the strain survived during 4 and 20 days storage, respectively. However, in the same heat treatment, there were recorded differences: the highest number of *C. jejuni* was obtained in microaerobe conditions and the lowest number by keeping the samples under aerobic conditions.
Although the refrigeration is used to control bacterial growth in foods, the researches proved that it can not be a substitute for safe handling and production measures and for proper cooking of meat.

Campylobacter germs are a common cause of food-borne outbreaks in the EU; in 2007, campylobacteriosis was the most frequently reported zoonotic disease in humans (EFSA, 2009). About 95% of the campylobacteriosis cases are caused by the species *C. jejuni*. The major risk factor for human *Campylobacter* infections is believed to be chicken meat (Boysen *et al*. 2007).

In laboratory studies, *Campylobacter* germs have been found to be sensitive to O_2 in concentration above 10%, while CO_2 was found to stimulate growth (Wingstrand *et al*., 2006). Campylobacters have an optimal growth at temperatures ranging between 37 and 42°C and they do not grow below 30°C; however, *C. jejuni* has been shown to display physiological activity at 4°C (Bhaduri and Cottrell, 2004; Solow *et al*., 2003).

The aim of the present study was to determine the survival of *C. jejuni* under different conditions of temperature and gas mixtures.

1. MATERIALS AND METHODS

In this study it was used *Campylobacter jejuni* strain ATCC 33291, which was subcultured onto Columbia blood agar (5 % sheep blood). The plates were incubated in microaerobic atmosphere with the following composition: oxygen 5%, carbon dioxide 10% and hydrogen 10%; the incubation temperature was 41.5°C. After 24 hours, from the obtained bacterial culture was made a bacterial suspension (in Brucella broth) with a density of 0.5 on McFarland scale; this density corresponds to about 10^6 cfu/ml. From this suspension were prepared serial dilutions; in the study it was used the dilution with about 10^5 cfu/ml.

The meat used in the experiments was represented by minced chicken carcass, due to the possibility of obtaining a good mixing of the sample. The meat was divided into three samples, each containing 300 g. Before inoculation, the meat was cultured to detect possible natural contamination with *Campylobacter*; no positive samples were found. Each sample was artificially contaminated with 3 ml of *Campylobacter* culture, the number of microorganism in the inoculum being about 10^5 cfu/ml; for a good homogenization, the samples were mixed using a stomacher.

From each sample, 10 g (considered blank) were collected in order to estimate the contamination of meat immediately after adding the culture of *Campylobacter*. Over the 10 g of sample were added 90 ml buffered peptone water; 0.1 ml of the initial suspension and decimal dilutions were spread onto the surface of plates containing modified charcoal cefoperazone deoxycholate agar (mCCDA). The plates were incubated at 41.5°C for 48 hours, in microaerobic atmosphere. After incubation, there were counted the typical colonies from each plate and there were selected five colonies for confirmation. The confirmation has been made by the examination of morphology and motility, by the detection of oxidase and by studying the growth at 25°C in microaerobic atmosphere and 41.5°C in aerobic atmosphere.

The first sample was divided in three subsamples, as follows: sample A_1 was incubated in vacuum atmosphere, sample B_1 was incubated in aerobic conditions and sample C_1 was incubated in a gas mixture containing 85% N_2, 10% CO_2 and 5% O_2. In the same way was done with the other two samples, resulting A_2, B_2, C_2 samples, respectively A_3, B_3, C_3 samples.

The samples A_1, B_1, C_1 were incubated at 37°C for 48 hours, the samples A_2, B_2, C_2 were incubated at 20°C for 4 days and the samples

A_3, B_3, C_3 were incubated at 4°C for 20 days. The samples A_1, B_1, C_1 were examined at 24 and 48 hours, the samples A_2, B_2, C_2 were examined at 1, 2 and 4 days, and the samples A_3, B_3, C_3 were examined at 2, 4, 10 and 20 days. The analysis of the samples was made in the same way as for the blank.

2. RESULTS AND DISCUSSIONS

At 37°C, the cell counts of *C. jejuni* strain increased regardless of atmospheric composition. In the sample incubated in microaerophlic conditions, *Campylobacter* growth was the best; this fact can be explained both by creating optimal conditions for the development of *Campylobacter* and by inhibiting competitive flora for which gas mixture was not favorable. Also, it is noticeable that vacuum atmosphere is more favorable to the growth of *Campylobacter* comparative with aerophilic atmosphere, which contains oxygen (Table 1, Fig. 1).

Table 1

The growth of *Campylobacter jejuni* at 37°C in: A_1 – vacuum atmosphere, B_1 – aerophilic atmosphere, C_1 – microaerophilic atmosphere

Sample	Nr. of *Campylobacter jejuni* (log10 cfu/10 g)		
	0 hours	24 hours	48 hours
A_1	3.97	6.12	7.38
B_1	3.97	4.06	4.16
C_1	3.97	6.98	8.56

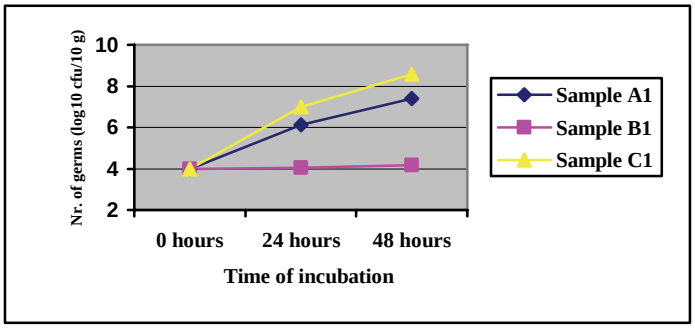

***Fig. 1.* The diagram of *Campylobacter jejuni* growth at 37°C in: A_1 – vacuum atmosphere, B_1 – aerophilic atmosphere, C_1 – microaerophilic atmosphere**

At 20°C and 4°C, the strain survived during 4 and 20 days of storage, respectively. Still, the survival of *Campylobacter jejuni* was better in the optimal gas atmosphere than in the other studied situations

(Tables 2, 3; Fig. 2, 3). The lowest number of *C. jejuni* germs was recorded in the plates incubated in aerophilic conditions (normal atmosphere), regardless the temperature.

Table 2

The growth of *Campylobacter jejuni* at 20°C in: A_2 – vacuum atmosphere, B_2 – aerophilic atmosphere, C_2 – microaerophilic atmosphere

Sample	Nr. of *Campylobacter jejuni* (log10 cfu/10 g)			
	0 hours	1 day	2 days	4 days
A_2	3.97	3.08	2.87	2.61
B_2	3.97	3.01	2.65	2.25
C_2	3.97	3.41	3.14	2.90

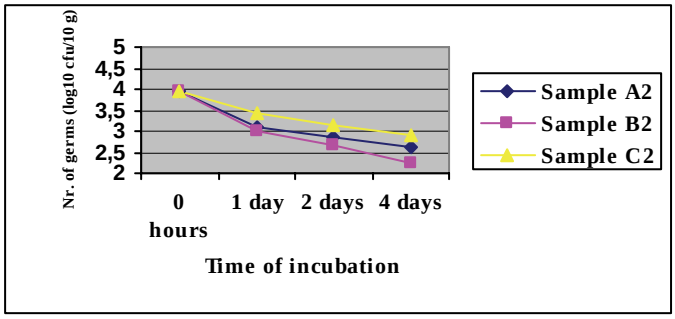

Fig. 2. **The diagram of *Campylobacter jejuni* growth at 20°C in: A_1 – vacuum atmosphere, B_1 – aerophilic atmosphere, C_1 – microaerophilic atmosphere**

Table 3

The growth of *Campylobacter jejuni* at 4°C in: A_3 – vacuum atmosphere, B_3 – aerophilic atmosphere, C_3 – microaerophilic atmosphere

Sample	Nr. of *Campylobacter jejuni* (log10 cfu/10 g)				
	0 hours	2 days	4 days	10 days	20 days
A_3	3.97	3.17	2.92	2.47	2.30
B_3	3.97	2.96	2.65	2.32	2.08
C_3	3.97	3.31	3.11	2.88	2.54

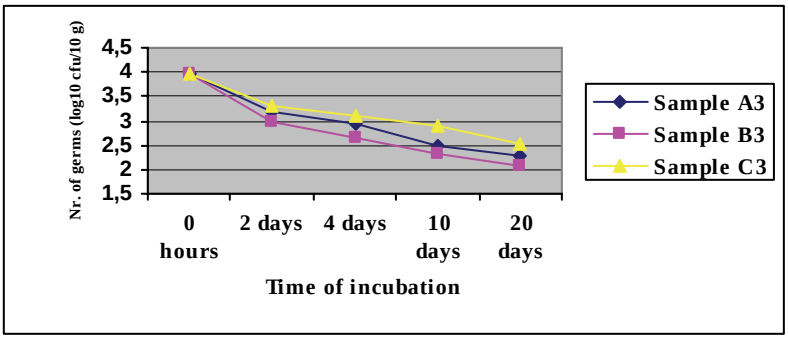

Fig. 3. **The diagram of *Campylobacter jejuni* growth at 4°C in: A_1 – vacuum atmosphere, B_1 – aerophilic atmosphere, C_1 – microaerophilic atmosphere**

Although the number of *C. jejuni* was reduced by freezing, these conditions still preserved enough microorganisms to produce human disease. This fact proves that refrigeration is not a safe method of disposing these bacteria and it cannot replace the hygiene measures during production and handling, as well as the proper cooking of the meat.

The oxygen in air is toxic for campylobacters and it is one of the most important factors implicated in the survival of these bacteria in different atmospheres.

In the present study, the competing flora was not investigated, but earlier studies have shown that during storage in atmospheric conditions mentioned before, bacterial flora will change from Gram negative aerobic bacteria to lactic acid bacteria and facultative anaerobic bacteria (Boysen *et. al*, 2007).

3. CONCLUSIONS

3.1. At 37°C, the cell counts of *C jejuni* strain increased, regardless of the atmospheric composition.

3.2. A significant proportion of *Campylobacter jejuni* survived in meat chicken samples maintained at 20°C and 4°C, regardless of the used gas mixtures.

3.3. Although the number of *C. jejuni* was reduced by freezing, meat samples still preserved enough bacteria to produce the disease in consumers.

3.4. The refrigeration is not a safe method of disposing these bacteria and it cannot replace hygiene measures during production and handling and proper cooking of meat.

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THE INFLUENCE OF FOUR SELECTIVE CULTURE MEDIA ON THE ISOLATION OF *CAMPYLOBACTER SPP.*

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SUMMARY

According to the E.U. reporting on zoonosis, germs of *Campylobacter* genus represent at present the main zoonotic agent involved in the appearance of gastroenteritis in humans.
Culture media for the isolation of *Campylobacter* usually contain various selective agents, designed to allow these bacteria to grow whilst suppressing the growth of other microorganisms. For example, cefoperazone, vancomycin and polymyxin B are antibiotics incorporated into *Campylobacter* culture media in order to inhibit the growth of competitor germs.
In this study, the selectivity of four *Campylobacter* culture media was studied by quantifying the growth of *Campylobacter* strains and the suppression of potential competitor microorganisms.
The used strains of *Campylobacter* (*C. coli* and *C. jejuni*) developed well on the four agars, there were not inhibited by antibiotic substances contained within selective media, while the flora of association was differently suppressed, depending on the used medium.
mCCD agar and Preston agar provided a better selectivity for *Campylobacter jejuni* and *Campylobacter coli* than Skirrow agar and Karmali agar. From the four tested selective media, the less selective was Skirrow agar. The selectivity of culture media can be attributed to the influence of basic medium, growth promoting additives and inhibitory supplements.

Campylobacteriosis represent an important public health problem in most areas of the world. According to the E.U. reporting on zoonosis, germs of *Campylobacter* genus represent at present the main zoonotic agent involved in the appearance of gastroenteritis in humans (EFSA, 2009). Thermophilic *Campylobacter* species, particularly *C. jejuni* and *C. coli*, are extremely important because of their harmful impact on humans (Park, 2002).

The isolation of campylobacters requires special selective techniques because of their microaerophilic nature and because these bacteria can be present very poorly in samples comparatively to competitors germs (Bârzoi and Apostu, 2002). Thereby, four selective media have been studied, as follows: modified Charcoal Cefoperazone Deoxycholate agar (mCCD agar), Karmali agar, Preston agar and Skirrow agar. All these media contain antibiotics to inhibit the association flora, thus creating conditions for the development of *Campylobacter* germs (Ronald *et al.*, 2006).

The aim of this study was to assess the effectiveness of four selective media and to determine which selective media provides the

best balance between the development of *Campylobacter* germs and the inhibition of competitor germs.

1. MATERIALS AND METHODS

Culture media for the isolation of *Campylobacter* usually contain various selective agents, designed to allow these bacteria to grow whilst suppressing the growth of other microorganisms (Martin *et al.*, 2002).

The composition of the used selective media is presented in table 1. All media were prepared from dehydrated culture media and the supplements required; all the components were recommended by Oxoid.

Table 1

Composition of the four selective media that were used for *Campylobacter* germs isolation

Constituent	Media			
	mCCDA agar	**Karmali agar**	**Skirrow agar**	**Preston agar**
Dehydrated culture media				
Nutrient broth no. 2	25.0 g	-	-	25.0 g
Blood agar base no. 2	-	-	27.5 g	-
Columbia agar base	-	39.0 g	-	-
Bacteriological charcoal	4.0 g	4.0 g	-	-
Casein hydrolysate	3.0 g	-	-	-
Sodium deoxycholate	1.0 g	-	-	-
Ferrous sulphate	0.25 g	-	-	-
Sodium pyruvate	0.25 g	-	-	-
Haemin	-	0.032 g	-	-
Agar	12 g	12 g	12 g	12 g
Supplements				
Sterile lysed defibrinated horse blood	-	-	50 ml	50 ml
Cefoperazone mg/l	32	32	-	-
Amphotericin B mg/l	10	-	-	-
Polymyxin B IU/l	-	-	2500	5000
Rifampicin mg/l	-	-	-	10
Trimethoprim mg/l	-	-	5	10
Cycloheximide mg/l	-	100	-	10
Sodium pyruvate mg/l	-	100	-	-
Vancomycin mg/l	-	20	10	-

The used food sample was minced chicken; this preference was due to the possibility of obtaining a good mixing of the sample.

Bacterial strains that were used were: *Campylobacter jejuni, Campylobacter coli, Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus*; these strains derived from previous isolations from different foods. The two strains of *Campylobacter spp*. were chosen because of their involvement in human pathology.

Campylobacter strains were subcultured onto Columbia blood agar (5 % sheep blood). The plates were incubated in microaerobic atmosphere with oxygen content of 5 %, carbon dioxide 10 % and hydrogen 10 %; the incubation temperature was 41.5°C. The non-*Campylobacter* strains were subcultured onto nutritive agar and incubated in aerobic condition at 37°C.

After 24 hours, from each bacterial culture were made bacterial suspensions with a density of 0.5 on McFarland scale (this density corresponds to about 10^6 cfu/ml); bacterial suspensions were performed in physiological saline solution. From this suspensions were prepared serial dilutions for each bacterial strain; in the study were used the dilutions with about 10^5 cfu/ml.

Over 25 g minced meat was added 1 ml of each bacterial suspension; for a good homogenization, the sample was mixed using a stomacher. Then, over the sample there were added 270 ml buffered peptone water, obtaining the dilution 10^{-1}; from this dilution, 10^{-2} dilution was prepared. 0.1 ml from each dilution was spread uniformly over the surfaces of two plates of each tested selective media. The plates prepared this way were incubated for 48 hours at 41.5°C in microaerobic atmosphere. After incubation, there were counted the typical colonies from each plate and there were selected five colonies for confirmation. The confirmation was made by examining the morphology and motility, by the detection of oxidase and by studying the growth at 25°C in microaerobic atmosphere and 41.5°C in aerobic atmosphere. The characteristics of *Campylobacter spp*. germs are presented in table 2.

Table 2

Characteristics of *Campylobacter spp.* germs

Morphology	Small curved bacilli
Motility	Characteristic
Micro-aerobic growth at 25°C	-
Aerobic growth at 41,5°C	-
Oxidase	+

The number of *Campylobacter spp*. germs was established based on the number of confirmed colonies and applying standardized formulas.

2. RESULTS AND DISCUSSIONS

In table 3 it is presented the total number of colonies on the four medium used, the number of typical colonies and the number and percentage of colonies confirmed as *Campylobacter spp*.

Table 3

Number of colonies grown on the four used selective media

Selective media	Total number of colonies			Number of typical colonies			Colonies positive confirmed	
	Plate 1 (10^{-1})	Plate 2 (10^{-2})	Σ	Plate 1 (10^{-1})	Plate 2 (10^{-2})	Σ	Nr.	%
mCCDA agar	77	9	86	77	9	86	86	100
Karmali agar	72	10	82	69	8	77	50	60.97
Skirrow agar	117	22	139	93	14	107	61	43.88
Preston agar	91	8	99	86	8	94	91	91.91

The selective media tested in this study were designed primarily to isolate *Campylobacter* species that grow optimally at 41.5°C. The two strains of *Campylobacter* used (*C. coli* and *C. jejuni*) developed well on the four agars and were not inhibited by antibiotic substances contained within selective media. However, it is noticeable that the highest percentage of positive confirmed colonies was recorded on mCCDA and Preston agars (100% and 91.91%, respectively), comparatively to Karmali and Skirrow agars (60.97% and 43.88%, respectively). These percentages can be explained based on the development of other bacteria on Karmali and Skirrow agars, which means a lower selectivity of these media. The differences between the four selective media used on the balance between stimulation of *Campylobacter* growth and inhibition competitive flora are presented in figure 1.

In case of Karmali and Skirrow agar, although the percentage of colonies confirmed as *Campylobacter* is relatively similar, Skirrow agar is the least selective, allowing the initial development of a large number of non-*Campylobacter* bacteria.

Culture plates were incubated under the same conditions of time, temperature, and atmosphere; therefore, the differences in selectivity between the media can be attributed to the influence of their three major

components: basal medium, growth promoting additives and inhibitory supplements.

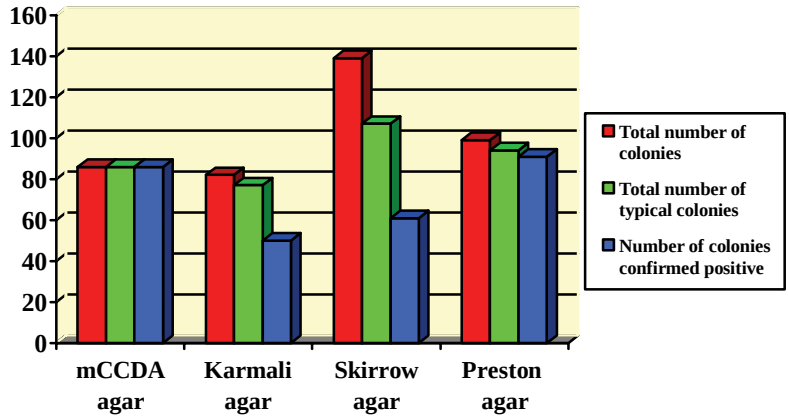

***Fig. 1.* – Total number of colonies, total number of typical colonies and number of colonies confirmed positive grown on the four used selective agars**

3. CONCLUSIONS

3.1. mCCD agar and Preston agar provided a better selectivity for *Campylobacter jejuni* and *Campylobacter coli* than Skirrow agar and Karmali agar.

3.2. From the four tested selective media, the less selective was Skirrow agar.

3.3. The selectivity between the different solid culture media can be attributed to the influence of basic medium, growth promoting additives and inhibitory supplements.

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ANTIMICROBIAL ACTIVITY OF EXTRACTS OBTAINED FROM SEA BUCKTHORN

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Keywords: sea buckthorn, alcoholic extract, polyphenols, antibacterial activity.

SUMMARY

The replacement of synthetic additives with plant polyphenols is nowadays an important scientific objective. Recent studies demonstrated that polyphenols extracted from leaves, fruits, seeds and roots of different plants can manifest an antimicrobial activity. Sea buckthorn fruits *(Hippophae rhamnoides)* contain important quantities of flavonoids and phenolic acids.
The purpose of this study was to determine the antibacterial capacity of polyphenols from sea buckthorn fruits (*Hippophae rhamnoides*).
The researches were performed upon 5 bacterial strains (*Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 11778) that were tested using diffusimetric method in order to evaluate their sensitivity/resistance to a polyphenolic extract obtained from sea buckthorn fruits.
The obtained results showed that the polyphenols extracted from sea buckthorn fruits have antibacterial properties. The most sensitive bacteria to the action of sea buckthorn polyphenols were *B. cereus* and *B. subtilis*. *S. typhimurium* and *S. aureus* presented intermediary inhibition areas to the action of sea buckthorn extract. *E. coli* was the most resistant bacteria to polyphenols extracted from sea buckthorn fruits.

The replacement of synthetic additives with plant polyphenols is nowadays an important scientific objective. Natural antioxidants are thought to be safe, as long as they are found in plants used frequently in human's diet, whereas synthetic additives are suspected to be responsible for many diseases. Polyphenols are found in all plants and in all parts of plants and they are associated with antioxidant properties. Recent studies demonstrated that polyphenols extracted from leaves, fruits, seeds and roots of different plants can manifest an antimicrobial activity (Puupponen-Pimia *et al.*, 2004; Taguri *et al.*, 2004; Ullah *et al.*, 2008).

Structurally, polyphenols are compounds that contain hydroxyl functional groups bound to benzene rings. Generally, all chemical compounds of this type possess an antimicrobial activity. Studies on different classes of polyphenols demonstrated that antimicrobial activity varies proportionally with the number of hydroxyl groups (Kazmi *et al.*, 1994; Krishnaveni and Srinivasa Rao, 2000; Manjunatha, 2006;

Scalbert, 1991). For instance, catechol and pyrogallol manifest a toxic action upon microorganisms (Ikigai *et al.*, 1993).

Antimicrobial activity of polyphenolic extracts obtained from plants can be explained on the following mechanisms of action:

- denaturation of proteins from cell membrane and soluble cytoplasmatic proteins (Tsuchiya *et al.*, 1996);
- inhibition of extracellular microbial enzymes and metabolisms and deprivation of the substrate required for microbial growth (Bell *et al.*, 1965);
- destruction of lipid double layer from cell membrane (Ikigai *et al.*, 1993).

Due to the conjugated action upon proteins and lipids, cell membrane is affected, leading to the egression of essential constituents for the bacterial cell (Min *et al.*, 2008).

Sea buckthorn fruits *(Hippophae rhamnoides)* contain important quantities of flavonoids and phenolic acids (Papuc *et al.*, 2009; Zeb, 2004; Zhao and Fuheng, 1997).

The purpose of this study was to determine the antibacterial capacity of polyphenols from sea buckthorn fruits (*Hippophae rhamnoides*).

1. MATERIALS AND METHODS

Dried fruits of sea buckthorn were powdered and then extracted in a Soxhlet extractor with ethanol at 60ºC. The obtained extracts were filtered in order to obtain particle free extracts and evaporated on a rotary evaporator. The obtained residues were dissolved in ethanol (Papuc *et al.*, 2008; Papuc *et al.*, 2009).

The total phenolic content was determined by mixing 10µl sea buckthorn extract with 2.5 ml 10% Folin Ciocalteu`s reagent (v/v) and 2.0 ml of 7.5% sodium carbonate solution. The reaction mixture was incubated at 45ºC for 40 min, and the absorbance was measured at 765 nm using an UV-VIS spectrophotometer. Tannic acid was used as standard phenol (Papuc *et al.*, 2009).

In order to test the antibacterial activity of the obtained extract, there were used 5 bacterial strains, as follows: *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 11778.

The bacterial strains were initially cultivated in nutritive broth for 24 hours at 37°C; after incubation, bacterial strains were diluted with

physiologic saline solution in order to obtain the value 0.5 on McFarland scale, this representing the equivalent of 10^6 colony forming units (cfu) / ml.

As solid culture medium it was used Mueller-Hinton agar, this one being recommended in case of antibiograms based on the diffusion of antibacterial substances in medium. In each Petri plate there were poured 12-15 ml agar, which was permitted to solidify.

For the experiments there were used 5 ml of sea buckthorn alcoholic extract in concentration of 50 ppm polyphenols/ml. To eliminate the possibility of obtaining false-positive reactions (due to alcohol), the alcohol was removed by evaporation. The remaining film was dissolved in 300 µl propylene glycol; 10 µl (8.3 µg polyphenols) of the obtained mixture were used to soak filter-paper rounds of 6 mm in diameter.

Also, in order to be sure that the propylene glycol used for dissolution of the film doesn't affect bacterial growth, there were used negative-controls (filter-paper rounds soaked with 10 µl propylene glycol). There were used also 3 positive-controls represented by gentamicin (CN), amoxycillin (AMX) and chloramphenicol (C) microtablets. For each bacterial strain there were used 2 Petri plates.

The plates were flooded with bacterial cultures and left at room temperature for 5 minutes; after that, the overflow was removed by absorption and the medium surface was dried in incubator for 15 minutes at 37°C. After this interval, on the medium surface there were put filter-paper rounds soaked with the sample extract, negative and positive controls. The plates were incubated for 24 hours at different temperatures, depending on the bacteria: 37°C for *Salmonella typhimurium, Escherichia coli* and *Staphylococcus aureus* and 30°C for *Bacillus subtilis* and *Bacillus cereus* (CLSI, 2006).

2. RESULTS AND DISCUSSIONS

The obtained results showed that sea buckthorn polyphenolic extract has the capacity to inhibit the development of studied bacterial strains, but this effect is different depending on the bacteria. Thus, the largest inhibition areas were recorded in case of *B. cereus* and *B. subtilis*, proving that on these two bacteria the extract has the highest antibacterial activity. On the other hand, on *E. coli* the extract had a low antibacterial activity; this bacteria was considered resistant to the used concentration of polyphenols. *S. typhimurium* şi *S. aureus* presented intermediary inhibition areas.

The obtained results are presented in table 1 şi fig. 1.

Table 1

The influence of sea buckthorn polyphenolic extract upon different bacterial strains - the diameter of inhibition area

Bacteria		Diameter of the inhibition area (mm)				
		Negative control	Sea buckthorn extract	Microtablet with antibiotic (positive control)		
				CN 10 µg	C 30 µg	AMX 25 µg
Bacillus subtilis	Plate 1	6	13	18	16	18
	Plate 2	6	13	19	16	18
Escherichia coli	Plate 1	6	8	11	14	10
	Plate 2	6	8	12	12	11
Salmonella typhimurium	Plate 1	6	9	12	24	13
	Plate 2	6	9	12	22	13
Staphylococcus aureus	Plate 1	6	9	18	20	12
	Plate 2	6	9	17	19	11
Bacillus cereus	Plate 1	6	13	16	14	6
	Plate 2	6	12	16	14	6

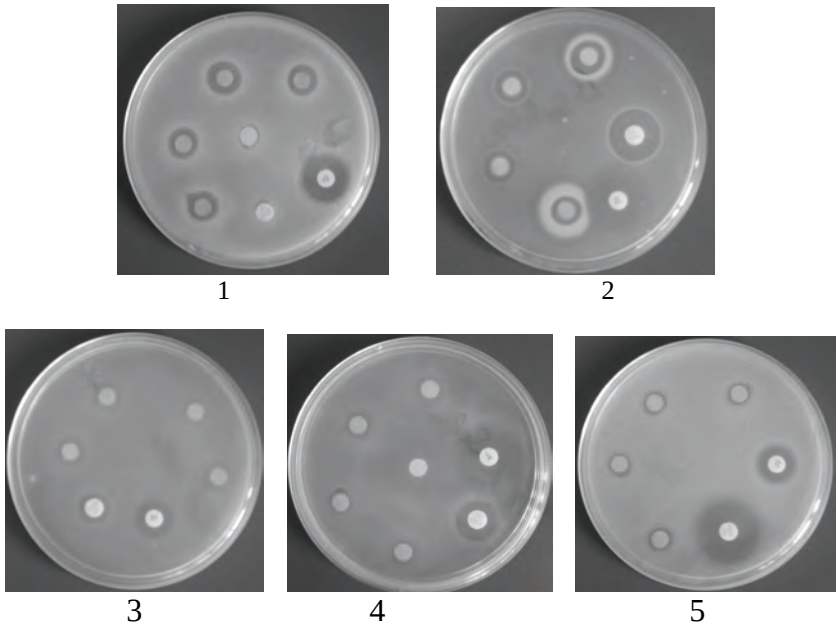

Fig. 1. **The influence of sea buckthorn polyphenolic extract upon different bacterial strains (1 – *B. cereus*; 2 – *B. subtilis*; 3 – *E. coli*; 4 – S. *typhimurium*; 5 – *S. aureus*)**

Globally, the inhibition areas produced by the samples were smaller then the ones produced by antibiotic microtablets; however, it is noticeable the influence of sea buckthorn extract upon studied bacteria, considering the relatively low quantity of active substance (8,3 µg)

comparatively to positive controls (gentamicin 10 µg, chloramphenicol 30 µg, amoxycillin 25 µg).

3. CONCLUSIONS

The polyphenols extracted from sea buckthorn fruits have antibacterial properties.

The most sensitive bacteria to the action of sea buckthorn polyphenols were *B. cereus* and *B. subtilis*.

S. typhimurium and *S. aureus* presented intermediary inhibition areas to the action of sea buckthorn extract.

E. coli was the most resistant bacteria to polyphenols extracted from sea buckthorn fruits.

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THE PROTECTIVE EFFECT OF SEA BUCKTHORN ALCOHOLIC EXTRACT UPON PROTEINS AND LIPIDS FROM REFRIGERATED BEEF AND PORK

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Keywords: sea buckthorn, polyphenols, proteins, lipids, refrigerated meat.

SUMMARY

Muscle tissue refrigeration evolution is characterized by biochemical reactions that affect proteins and lipids.
The purpose of the researches presented in this paper was to investigate the effects of sea buckthorn alcoholic extract on proteins and lipids from refrigerated beef and pork muscle. The proteolytic degradation was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the course of lipid peroxidation was monitored by measuring conjugated dienes (CD), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) levels.
The sea buckthorn polyphenolic extract had a protective action upon proteins from refrigerated beef and pork. Sea buckthorn polyphenols protected unsaturated fatty acids from refrigerated beef and pork against lipid peroxidation. Due to these actions, sea buckthorn polyphenols can be used in food industry to protect minced meat against proteolytic and oxidative processes.

Refrigeration is an important process for prolonged preservation of fresh meat. Muscle tissue refrigeration evolution is characterized by biochemical reactions that affect proteins and lipids. A proteolytic degradation of myofibrillar and connective tissue components was observed in tissues from different species of animals (Hopkins and Thompson, 2002; Ogata *et al.*, 1998; Olson *et al*., 1977; Tseng *et al*., 2002). Two major intracellular degradative pathways have been involved in this degradation: a lysosomal way including cathepsic proteases and a cytosolic calcium-dependent way with calpains (Ladrat *et al*., 2003; Matsuishi and Okitani, 1997; Olson *et al*., 1977).

As long as the meat is stored in the cold in large cuts, lipid oxidation is very slow, whereas chopping, mincing or warming the muscle tissue cause a high rate of peroxidation. Muscle disintegration results cause a high rate of peroxidation because in this process a low but significant release of highly unsaturated membrane phospholipids and Fe^{2+} ions from myoglobin was observed. Proteolytic degradation of proteins and oxidative processes in meat are the most important factors responsible

for quality deterioration, including flavor, color and nutritive value (Petron *et al*., 2007).

In order to slow down the oxidative processes, in minced meat are added synthetic compounds with antioxidant action. Studies performed in the last years demonstrated that synthetic antioxidants are toxic and can affect consumers' health. The replacement of synthetic antioxidants with plant polyphenols represents nowadays a high interest objective because they have an antioxidant activity similar to butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) synthetic antioxidants, they are not toxic and, moreover, they have a benefic effect upon consumers' health. Plant polyphenols, especially flavonoids, inhibit the activity of lipoxygenase enzyme and the chelation of Fe^{2+} ion which concurs to the initiation of lipid peroxidation process (Irwandi and Che Man, 2000; Ramadan *et al*., 2003; Stoilova *et al*., 2008).

Sea buckthorn fruits (*Hippophae rhamnoides*) contain high quantities of flavonoids and phenolic acids with antioxidant activity (Zeb, 2004; Zhao and Fuheng, 1997). Several *in vitro* studies demonstrated that sea buckthorn polyphenols have the ability to scavenge reactive oxygen species and to inhibit lipid peroxidation process (Papuc *et al*., 2008; Papuc *et al*., 2009).

The purpose of the researches presented in this paper was to investigate the effects of sea buckthorn alcoholic extract on proteins and lipids from refrigerated beef and pork muscle. The proteolytic degradation was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the course of lipid peroxidation was monitored by measuring conjugated dienes (CD), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) levels.

1. MATERIALS AND METHODS

Preparation of Hippophae rhamnoides alcoholic extract. Crude polyphenols of sea buckthorn were extracted according with the method described by Papuc *et al*. (2008).

Preparation of meat samples. For the researches it was used beef and pork thigh; meat samples were minced using a blender. Different quantities of sea buckthorn alcoholic extract were put in clean Petri plates; after the evaporation of alcohol, in each Petri plate were added 5 g of meat. The samples were homogenized, covered with polyethylene sheet and then stored at 4°C for 6 days. Control samples were prepared the same way, with the difference that no sea buckthorn extract was used.

Obtaining protein extracts. Muscle samples were mortared and then subdued to an extraction with deionized water (5°C) in a ratio of 1:10 (w:v). The obtained extracts were filtered, centrifuged and analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to detect proteolytic changes in red muscle during refrigerated storage. The SDS-PAGE system consisting of a 10% acrylamide gel was run following the procedure described by Srinivasan *et al*. (1997). The homogenate was diluted with 1:1 (v:v) with the sample buffer containing 4% SDS, 0.125M Tris (pH 6.8), 20% glycerol and 10% β-mercaptoethanol.

The extraction of total lipids. Total lipids from meat were extracted with a chloroform/methanol/water mixture (40:28:32; v/v/v). After filtration, 0.98% potassium chloride was added; the mixture was then centrifuged. The obtained chloroformic layer was used for the determination of conjugated dienes and lipid peroxides (Blingh and Dyer, 1959).

Conjugated dienes assay (CD). 0.1 ml lipid extract was diluted with 2 ml of cyclohexane and then absorbance was measured at 233nm against blank (cyclohexane), using a Jasco V650 UV-VIS-NIR spectrophotometer. The inhibition of conjugated dienes formation was expressed as %Inhibition, according to the relation:

$$\%Inhibition = (A_{control} - A_{sample}) x\ 100/A_{control},$$

where, $A_{control}$ – absorbance of the blank (without sea buckthorn extract);

A_{sample} – absorbance of the sample.

Peroxide value assay (PV). 0.02g sea buckthorn extract was dissolved in 9.8 ml methanol:chloroform mixture (30:70, v/v) and 0.1 ml ammonium thiocyanate 300 g/l. After homogenization there was added 0.1 ml ferrous chloride prepared in hydrochloric acid. The red color of the mixture was estimated at 501 nm against blank consisting in a methanol:chloroform mixture (70:30, v/v) (Romero *et* al., 2008). The obtained results were expressed as %Inhibition.

Thiobarbituric acid reactive substances (TBARS) assay. To 100 mg water-methanol layer of extracts were added 100 µl BHA 36 g/l, 2 ml thiobarbituric acid (TBA) / trichloroacetic acid (TCA) mixture (20 mM TBA in 150 g/l TCA). The mixture was warmed up in a water bath at 90°C for 15 min. and then cooled at room temperature. After adding 2 ml of chloroform, the mixture was centrifuged at 1000xg. The absorbance of chloroformic layer was estimated at 532 nm against a

blank consisting in chloroform (Romero *et* al., 2008). The obtained results were expressed as %Inhibition.

2. RESULTS AND DISCUSSIONS

Polyacrylamide gel electrophoresis. The electrophoretic analysis did not revealed major proteolytic modifications of proteins electrophoretic distribution after 6 days of refrigeration. Generally, the preponderant proteins (actin and myosin) proved to be resistant to proteolysis. The only observed differences between control samples and sea buckthorn-treated samples were at the level of electrophoretic distribution of small molecular weight proteins (Fig. 1). Thus, in control samples (both beef and pork muscle) there were observed 2 supplemental stripes, corresponding to the appearance of small molecular weight proteins pursuant to proteolytic processes. These stripes were not observed in case of samples treated with sea buckthorn polyphenols, which can indicate their protective action towards proteolytic enzymes.

Many endogenous enzymes could cause the proteolytic changes, but calpain and cathepsin may have played a major role in producing the observed changes in the control samples.

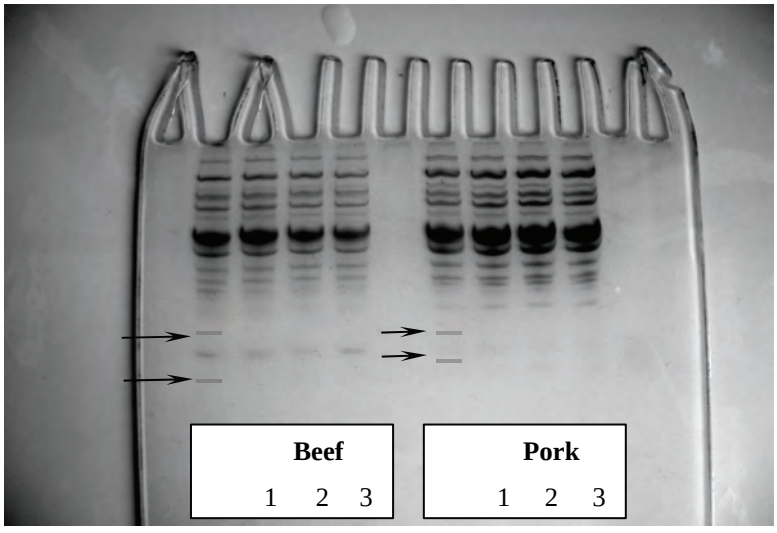

Fig. 1. **Electrophoretic analysis of proteolytic changes in beef and pork muscle during refrigeration for 6 days at 4°C (Comassie blue staining): 1 – control; 2 – sample treated with 0.1 ml of extract; 3 – sample treated with 0.2 ml of extract; 4 – sample treated with 0.3 ml of extract**

The formation of conjugated dienes (CD). The effect of sea buckthorn extract upon conjugated dienes formation process is presented in table 1. The obtained results demonstrate that sea buckthorn polyphenols inhibited conjugated dienes formation process both in pork and beef muscle refrigerated at 4°C for 6 days. The increase of polyphenols quantity leaded to the increase of lipid peroxidation process inhibition. The highest inhibition was observed when 0.3 ml sea buckthorn alcoholic extract were used (48.64% for pork and 50.90% for beef). The results were expressed as mean values of 3 determinations.

Table 1

The inhibitory effect of Sea buckthorn polyphenols upon lipid peroxidation process in pork and beef muscle refrigerated at 4°C for 6 days

Sea buckthorn alcoholic extract (ml)	%Inhibition CD		%Inhibition PV		%Inhibition TBARS	
	Pork	Beef	Pork	Beef	Pork	Beef
0.1	29.72	18.18	21.23	21.45	8.82	19.04
0.2	36.48	40.00	30.97	29.81	26.47	33.33
0.3	48.64	50.90	41.59	41.45	58.82	42.85

The formation of lipid peroxides (PV). The obtained results are similar to the ones for conjugated dienes. Thus, the highest inhibition percentage was recorded when 0.3 ml sea buckthorn polyphenolic extract were used (41.59% for pork and 41.45% for beef); the lowest inhibition percentage was recorded when 0.1 ml extract was used (21.23% for pork and 21.45% for beef) (table 1). The results were expressed as mean values of 3 determinations.

The formation of thiobarbituric acid reactive substances (TBARS). With thiobarbituric acid react lipid peroxidation secondary products (aldehydes and ketones), which modify lipids' smell and taste. After 6 days of refrigeration at 4°C, the lowest level of these compounds was recorded in meat samples treated with sea buckthorn polyphenols. Thus, in the samples treated with 0.3 ml of extract, the formation of aldehydes and ketones was inhibited with 58.82% in pork and with 42.85% in beef. Also, for the other two quantities of extract (0.1 ml and 0.2 ml), the obtained values were appreciable (table 1). The results were expressed as mean values of 3 determinations.

3. CONCLUSIONS

The sea buckthorn polyphenolic extract has a protective action upon proteins from refrigerated beef and pork; this property is attributed to the inhibitory action upon endogenous proteases present in the muscles.

Sea buckthorn polyphenols protect unsaturated fatty acids from refrigerated beef and pork against lipid peroxidation. The inhibitory effect of polyphenols upon lipid peroxidation process explains the decrease of the content in conjugated dienes, lipid peroxides and thiobarbituric acid reactive substances in case of the samples treated with sea buckthorn alcoholic extract.

Sea buckthorn polyphenols can be used in food industry to protect minced meat against proteolytic and oxidative processes.

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SCIENTIFIC ACADEMIC RESEARCH IN THE PRESENT INFORMATIONAL CONTEXT

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Key words: academic research, applied research, informational context, knowledge, scientific output

SUMMARY

The scientific research processes currently carried out in academic environments are undergoing a period of radical changes, being increasingly brought into question the role of academic scientific research in society and the directions of development that it should follow. In this development a key role is played by the equilibrium of the relationship between society's expectations on academic research and the informational background where it is placed. Scientific research conducted in universities is and will continue to be a primary source of information and knowledge, without which no other form of research can survive. The new knowledge obtained through scientific research process is the basis of the development and the foundation of social and historical progress, and of the human prosperity.

1. GENERAL CONTEXT OF UNIVERSITY SCIENTIFIC RESEARCH

1.1 Conceptual considerations on research

The act of knowledge was marked out as a primary feature, fundamental, of the man, and was born from quest and implicitly from discovery, enriched the man by acquiring knowledge, converting itself into a fundamental vector of progress of humanity. Today, the new knowledge gained from scientific research processes, is the basis of development and at the same time directions for strengthening an economy based on science, being constituted as the basis of social and historical progress and of the human prosperity.

Whatever the objectives of scientific research may be, the discovery of new phenomena, new causes of things or phenomena, laws and principles governing relations between phenomena, things, people, development of new techniques and technologies, innovation, improvement and efficiency in production, the finality of research processes produces new knowledge, generates the evolution of the system of human knowledge, drawing the development horizon of society. Scientific research, simplifying, has an idea as a starting point. The desire to prove something, to define, to delimit, to deepen, leads to study. Here should be seen an intimate connection that is established

from the outset between the theories and research methods - the theoretical-explanatory constructions, recording facts and demonstration of hypothesis. Research, in its broadest meaning is a scientific or critical inquiry carried out for discovery and interpretation of the facts or assumptions enunciated by an individual or group of individuals. Limiting the concept, scientific research is necessarily based on the completion of scientific methods and provides information and theories aimed at explaining the nature and properties of the surrounding world. It makes possible the emergence of practical explanations, without necessarily leading to them.

Regarding the ethics of research, this requires "a set of coherent principles and procedures across all research disciplines, describing, in the same time, an educational side available to all who participate in research projects implementation" [1]. Acceptance of a research project should lead to a clear ethical reflection on research and involves a continuous surveillance.

On research methods, they are not physically defined operations, but operators, means to pass knowledge from one state to another, the transformation produced by using the same methodology operator, maintaining the same logical structure, whatever the specific content of knowledge that applies may be. It establishes here a balance between the *observations* gained from research studies and *experiments*. Experimentation implies the existence of a hypothesis. This will be subject to examination of the facts. The research result aims to highlight the link between facts and foreseeable consequences of the hypothesis.

The link between theory, fact / hypothesis and experiment, however, involves a technique that implies a degree of technicality. With the risk of recurrence, here I consider again the technique characters transposition in various aspects of human practice, originally separated from technical work itself. Among other things, this reflects a desire to preserve the objectivity of the study, research. But even this technicality can be problematic for accuracy, correctness, appropriateness of the study. Besides a genuine technicality there may be a pseudo-technicality. I include here the technicality of essentially scientific thinking and the avoidance of its attachment to an ideology.

In scientific practice there is a language technique, one of the processes of observation and a third of applied processes[2]. In particular,

[1] www.cncsis.ro/includes/ghid_dreapta.htm Accesat 2.09.2009

[2] GRANGER, Gilles-Gaston. *Pour la connaissance philosophique.* Paris: Odile Jacob, 1988, pp. 135-140.

I discuss the technicality of observation processes, more generally, of establishing the facts. Scientific thinking cannot fully rely on the registration of phenomena, passively, just as it receives. There must be an organizational code of establishing the facts. In addition to these, it has to be taken into account also the language technique. Avoiding a common language is essentially required to avoid the trap of stereotypes. It is necessary to keep a certain distance from the common language, to allow the expression and development of knowledge, science. Evolution involves the singularity of specialized language, as a reflection of differentiation from old uniformalizing structures. Specialized language is evidence of autonomy of a discipline.

Scientific research - a procedural complex

Scientific research consists of a set of dynamic processes taking place on the trajectory of theory and practice, design and application, and distributed on several subdividing procedural directions:

■ Processes of fundamental research (basic) - aimed at determining new theories and laws governing natural or social phenomena;

■ Processes of applied research (investigation) - aimed at developing new techniques and technologies;

■ Developmental research processes (industrial) - aimed at the improvement and development of production, increasing economic efficiency.

Any process of scientific research must be subordinate to a certain type of scientific reasoning which is adapted to the specificities of each area of knowledge which is investigating. Specialized literature defines five concrete steps[3] to conduct scientific reasoning in any field it applies:

■ choosing and setting objectives or research topic;

■ formulating hypotheses;

■ choice of model to conduct scientific reasoning;

■ gathering, analyzing and comparing the results of scientific research;

■ drawing conclusions stemming from the investigations.

These steps can be grouped into three main stages[4], which meet the formal structure of a logical reasoning, steps that are taken by any scientific research activity:

[3] ENĂCHESCU, Constantin. *Tratat de teoria cercetării ştiinţifice.* Iaşi: Editura Polirom, 2005, p. 233.

[4] Idem op. cit.

■ general phase - which involves choosing and setting objectives or research topic and formulate hypotheses, from which is set out scientific reasoning;

■ particular stage - is exactly the research phase, operating with the object of research to prove the assumptions made;

■ conclusions' stage - getting agreement or disagreement between the assumptions and conclusions, consequently checking the results

When the discovery outlined following the scientific research is objectified, it is considered that the research has achieved its purpose and ends

Basic research (also called basic research or pure research) has as main objective the discovery of new information but also defining the theoretical meanings of existing things or simply of assumptions. It is an exploratory activity and often arises from the curiosity of researchers and their intuition. As such, it is often carried out without regard to any practical result, though sometimes it happens that basic research reveals unexpected practical results. The term "fundamental" reflects indeed also the theory that this type of research provides a starting point, a cornerstone for applied research. But often there is no guarantee of its applicability in the short term (consequently there can be no question of any technology transfer), as a result most researchers find hardly funding for such studies, except for university research programmes launched at a governmental level in each country.

Although basic research has traditionally been considered an activity that precedes applied research, and this one, in turn, precedes technology transfer, today, these discussions have become increasingly less clear. Today we meet a mix of research activities and technology transfer. This is evident especially in areas such as medicine, biotechnology and electronics, where fundamental discoveries may arise over new product development activities, as in these areas there are many cases of cooperation of public and private research funds.

1.3 Research information and documentation

To understand the role of research information and documentation phase, we must take into account the main stages of scientific research:

Define research topics;

Formulating hypotheses;

Establishing conceptual and operational definitions;

Gathering data;

Data analysis;

Tests, measurements, correcting assumptions;

Findings and new scientific definitions.

All steps above include an extensive documentary research and information activity, and the last of these is the publication activity (preparing articles, presenting papers, etc.) as part of scientific communication. We can thus say that the scientific information and documentation are the basic ingredients of research activities.

A well-documented assumption creates the premises of a scientific prediction and accuracy of tests, measurements and scientific findings will validate or invalidate the prediction. A new working hypothesis will be raised and this will lead to a scientific and more realistic prediction. Based on information and documents well selected, this process can be reduced regarding financial and time efforts.

The main purpose of research is to produce new knowledge. Scientific papers, such as articles in specialized journals, proceedings, articles in reference works, scientific blogs, Institutional Repositories, act as lucrative form of this new knowledge. Considering the type of "knowledge production", research can be divided into:

Exploratory research: that which structures and identifies new issues;

Constructive research: one that develops solutions to mooted questions;

Empirical research: testing the feasibility of a solution using empirical evidence (very often encountered in medicine, leading even to develop a new sub-branch "evidence-based medicine").

1.4 Results of research - Scientific publication

Academic publishing (scientific) describes a system that uses necessarily a formalized subsystem of scientific review and makes information available for large academic audiences. This system varies relating to results, organization and competences from one scientific domain to another. Most scientific papers are published in journals or books, but also in encyclopaedias or papers of conferences. In this editorial field, the term STM is a practical abbreviation of specialized literature in Science, Technology and Medicine and often found in professionals' language, in the scientific documentation field.

Each research area has its own scientific journals that reflect research activity in the respective field, but often we meet also journals dealing with interdisciplinary topics. Thus we meet also specialized publishing houses on certain scientific topics.

Today, publishing industry undergoes major changes, making the transition from print to the digital format. Operating models of this industry have changed radically, and this will continue for many years

from now on. We are not discussing only about the current content of publications, but also about their archives and preservation methods over time.

A particularly important aspect of this industry is the approach to open access to information regime, which actually means equal access for all people to scientific information. There are two models for open access to information. One is given when a publisher decides to offer free access to information published by him, and the second model also called open archives refers to the situation when an author or an organization producing information, choose to distribute for free (eliminating the role of editor) to all concerned. The second model is exposed to a high risk of proliferation of altered information, scientific unverified and which may contain important errors.

Anything addressing the subject of electronic sources of scientific information should consider their link with the academic environment in general and the academic research in particular. It remains to be demonstrated whether this link is a direct one and if enriched access to electronic sources of scientific information necessarily leads to increased performance in university research.

2. University research in the current relationship with society context

Contemporary university research is growing in a whole new social context, and having currently strong contacts with other institutions such as private institutions.

In research, collaborations - more or less organized - became a phenomenon of crucial importance for scientific practice. It is important to say that although there was always cooperation in scientific field, we are now witnessing a major change in this respect. Thus, today we hardly find individual results in medicine, natural sciences or engineering. Instead, teams (often international) and research networks are those that produce the most notable results. They are today the most industrious "molecules" producing scientific knowledge. Scientific perspective must be broader and include a micro and macro aspects of the research, so that we can speak of accepted results with a socio-cognitive value.

To better understand the relationship between university and society, we must realize the possible academic society policies on academic research. I will point out the following possible policies addressed in conjunction with the research:

■ ***Federalist policy*** requires that university research be integrated into the rest of society, playing a role of interface between the university and the social environment, reinforcing permanently restructuring process of the university system. Most researchers who examine the link between academic research and university environment embrace this position. We remark this approach in scientific journals such as *Technology Analysis & Strategic Management, R & D Management and Research Policy*. From their perspective, university research is seen as one of the many components of socio-economic development and should be managed, organized and evaluated so as to increase its social usefulness.

■ ***Internationalist policy*** supports the idea that university research is assimilated by the society, subordinate to it. Research development is organized by researchers as a one-sided answer to social processes. In this way university research is seen no more, no less that one of the many other cultural and educational practices of society. There is no distinction between university research and other modes of knowledge production. Social interests not only influence the choice of research themes, but also the methods and scientific validations. This approach is reflected in scientific journals as *Science, Technology & Human Values* and *Social Studies of Science*. But beyond the abstract views of supporters of this approach, academic research has produced scientific knowledge impossible to bring to the surface via other forms of cognitive production.

■ ***Sovereign policy*** requires that the university is a unique institution represented by a social system of collegiality. The agent of change is always within the institution and this system is threatened by anti-science and relativism trends. This approach implies a dependency of university research of an autonomous system of science policies. Disciplinary research is developing as a result of internal scientific procedures that cannot be influenced by practical utility objectives of research. All the university's internal mechanisms must be set such that to protect the autonomy of university research.

■ ***Isolationist policy*** requires that university research is a unique institution for the production of scientific knowledge. Trends that support research activity management as well as the ones that support marketing and industrialization of research knowledge are true threats to it. From this point of view is required to maintain the autonomy of decisions on university research and a minimum interference with the society.

CONCLUSIONS

1. The results of scientific research with practical application induce transformations in society, determines appreciation and respect for research, research need no longer being questioned in any area of existence. From this perspective it is necessary to produce shifts of resources, adoption of incentive regulations at the governmental level and governmental or nongovernmental bodies authorised in the research field, to mobilize in a synergistic way to achieve policies coherence on research, being in research phenomenon service.

2. In Romania, accredited higher education institutions and their structures, involving legislative and executive bodies, are included in the research-development system of national interest. Also, the legal framework underlying the organization and functioning of the Romanian higher education system, legislative and executive steps of our country, to create and develop an optimal framework for research and development activities to synchronize with the policies and strategies of European Research Area, credit such important institutions of knowledge system -universities, with specific tasks in the production of new knowledge - scientific research - transforming them into the determinants of knowledge in the Romanian space.

3. One of the major objectives of research policy today must be represented by developing a uniform system that contains relevant information for the following stages of a research project: financing, research practice, scientific results ("research output" - represented by publications, patents, technology transfer). To achieve this objective it is necessary to develop a theoretical framework to guide the reporting of research results.

4. Each of the university system approaches provides interesting hypotheses, but individually they are too restrictive for what means today's university research. Therefore, we must retain a balance when talking about both academic research developments, the informational context in which it is conducted, and the results expected from it.

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A BETTER USE OF INDIGENOVS PLANTS FOR GETTING BIOSTIMULATIVE FOOD

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Keywords: Cichoryum intybus, aromatic plants, biostimulative soft drinks, inuline (polyfructozan).

Abstract: This paper presents a way of better yield for the Cichorium intybus (mainly), blended with some indigenous medicinal and aromatic plants in order to obtain a flavored soluble natural concentrate that can successfully be used within the sanogenetic nutrition as biostimulative soft drinks.
The special rich chemical composition of chicory: inuline, glycoides, poliphenols, sugars, proteins, lipids, superior terpenoides, vitamins (C, B1, B2, B5), minerals (K, Na, Mg, P, Fe) it is the main reason for its use in the food and pharmaceutical industries.
The natural concentrate of chicory, blended with medicinal and aromatic plants, it has a trophic, biostimulative, energizing, and choleretic action, and it can be used for the preparation of soft drink, fruit juice, syrupus; it has a delicious taste and the pleasant flavor is given by the roasted chicory, medicinal and aromatic plants within the product recipe.
The technological process doesn't involve much energy consumption, it is simple and economical and can be performed using the current equipment of the food industry; it doesn't require a special previous preparation and it may be easily controlled concerning the quality of the raw material and finished product during the technological flow.
The industrial concentrate production enables the superior capitalization of some indigenous vegetal raw materials at high quality, high taste, and low cost as food products.

INTRODUCTION

In the last years the preoccupation with the development of the technologies for getting some substitutes for coffee or of the modified coffee considerably increased world widely, this was increasing the interest for chicory and other substitutes of the natural coffee.

The very special and interesting chemical composition of the chicory (*Cichorium intybus*), containing, besides a high content of inuline (polyfructozan), biter coumarinic glycoides, superior terpenoids, phenolic acids, proteins, fats, and vitamins shows a large range of compounds with potential to be used in alimentation or in the pharmaceutic industry, that added more interest concerning this plant.

This study, that is grounded the invention of the professors Paul Ştefănescu and Elena Ştefănescu (from UBB), it relates to a natural concentrate for refreshments, established from soluble extract of roasted chicory, water, mixed with a palette of indigenous medicinal and aromatic plants.

The soluble chicory concentrate, associated with plants natural extracts, it confers to the drink the qualities mentioned above and the ecological self preservation.

MATERIALS AND METHODS (BIOLOGIC MATERIAL USED)

The natural concentrate of chicory, medicinal and aromatic plants is made with a trophic, biostimulating, energizing, and coleretical action (it stimulate the secretion of bile). The concentrate can be used per se, blended with other alimentary products (fruit juices, syrupus, milk, creams etc.) or solved in mineral water or soda, fruit juice etc.

The aromatic plant contained in the product's receipt have pharmaco-dynamic actions: they reduce the thirst sensation, stimulate the gastric juice secretion, so that a lasting cure it concurs for the organism invigoration, for an improvement of its general state.

The technologic process, simple, not costly from the technical or economical point of view, it can be achieved with the current used equipment in industry, without a long term previous preparation, allowing an exact and efficient quality control on the technological flow, to the raw material or end product.

The concentrate it is obtained from the following raw materials:

- roasted chicory as a hydrosoluble natural concentrate of roasted chicory,
- crystallized sugar or *sweeteners*,
- alimentary citric acid,
- alcoholic extract from a mixture of indigenous aromatic and medicinal plants,
- ethanol 96;
- drinkable water or distilled water.

RESULTS AND DISCUSSION

In the next table are shown the organoleptical and physical-chemical characteristics of the concentrate of chicory and indigenous medicinal and aromatic plants:

A. Organoleptical Characteristics					
crt.no.	**Specification**				
1.	Aspect	Liquid opaque, ropy, sticky, without fermentation or mouldiness evidence			
2.	Color	Dark brown			
3.	Taste – Flavor	Specific: sweet-bitterish-sourish with a predominant flavor of caramel-citric and mint, blended in a very pleasant aroma			
4.	Precipitate	Free			
B. Physical-chemical Characteristics:					
No.	**Specification**	**M.U.**	**Values**		
			average	minimum	maximum
1.	Dry stuff	%	72.0	70.2	72.7
2.	Carbon hydrate, out of which	% d.s.	44.0	40.8	46.4
	Fructose	% d.s.	21.0	17.4	25.
	Sucrose	% d.s.	18.0	12.0	28.0
3.	Aminoacids-amide	% d.s.	13.0	11.3	16.6
4.	Betaine	mg % d.s.	1.0	traces	2.0
5.	Mineral compounds out of which: Potassium Calcium	% d.s. % d.s.	0.8 0.2	0.5 0.1	1.0 0.3
6.	Bitterish index	%	65.0	45.0	70.0
7.	Solubility in water at 70°C	%	99.0	97.0	100.0

CONCLUSIONS

The concentrate is obtained in conditions of economic efficiency, from a raw material easy to find, and it allows the obtaining of a various range of refreshments drinks having good gustative properties and a refreshing, energizing action, balancing the ions exchange at the cellular level, diuretic, coleretical and action of increasing the motility, carminative action, stimulating the metabolizing process of lipids, methionine and creathinine, with a good stability in time.

The concentrate defines itself through the following physiological and therapeutic effects:

It is an energizer due to the high content of sugars;

It has an action of increasing the motility and a hydrocoleretical action due to the esculine and its by-products;

It has hepatic-renal, diuretic, and depurative properties due to the phenolic acids and pepsids from the chicory;

It has a stimulating action for the gall bladder and intestinal smooth muscles;

It is not toxic;

It has a vitaminize action due to the vitamin C from the used indigenous medicine plants;

It is aseptic and antiperspirant, quality given by the fluid extract from the used indigenous medicine plants;

It stimulates the detoxification of nitrogen from the nucleotides catabolism or from urea, due to the alantoine content and of the betaine traces from the sweeteners;

The natural extracts from the medicinal and aromatic plants give to the product carminative, stomahical and lightly aseptic qualities.

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LEGISLATIVE BASIS OF THE PRIVATE VETERINARY PRACTICE IN BULGARIA

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KEY WORDS: private veterinary practice, clinics, dispensaries.

SUMMARY

In this work the legislative basis concerning the formation and the development of the private veterinary practice was investigated for the period from 1878 to 2008/2009.
The division of the local veterinary units and the allocation of the veterinary clinics and dispensaries according to the registered veterinary practitioners, were investigated in every administrative region in Bulgaria. The reflection of the tendency of feminization of the profession was studied regarding the private practice. Conclusions and suggestions were made for the elaboration of the private veterinary practice.

OBJECTIVES OF THE STUDY

With this work we aimed to investigate the formation and development of the private veterinary practice during the different stages of social and economic state of the country from 1878 to 2008/09. For this purpose we set up ourselves the following tasks:

- To examine the legislative based opportunities for having a private practice.
- To investigate at the present stage of time the allocation and the sufficiency of the private veterinarians in the administrative regions in the country.
- To determine the women:men proportion of the veterinarians with private practice according the tendency of feminization of the profession.

MATERIAL AND METHODS

An analysis of the legislative basis (veterinary legislation) was made which pointed the different stages from the formation of the private veterinary practice to its present state.

The methods used in the analysis were: retrospective analysis, descriptive analysis. Data were also gathered by the means of questionnaire. The reliability of the results for the present allocation of the private veterinary surgeons was guarantied by the representativeness of the data received from 25 of all 28 administrative regions in the country.

Statistical methods for processing the data were used as well.

RESULTS AND DISCUSSION

The historical development of the private veterinary practice passed through the following periods:

- 1878-1924 – Private veterinary practice after the Liberation of Bulgaria.

Sanitary act (1889), Act of the sanitary-veterinary police (1898), Act of the sanitary-veterinary service and police (1906) – these acts give the permission to the veterinarians to be either state officers or "free practitioners" after recognition of their professional qualification. Very few veterinary surgeons (both foreigners and Bulgarians, graduated abroad) and technicians take the chance for having a private practice because of their own insufficient number for serving the animal husbandry and the big number of unoccupied posts in the state and regional services on the other hand.

- 1924-1944 – Private veterinary practice during the period of Public veterinary medicine.

Act of the sanitary-veterinary service (1924) – after receiving a license for professional practice, the veterinary surgeons have the right to choose whether they prefer working on state service posts or if not so – working as free practitioners. The veterinary technicians are given the permission for "limited free practice" under special circumstance and control of the regional veterinary surgeon. The majority of the veterinary specialists prefer the budget posts instead of private practice because of the significant (social and material) stimuli offered by the state and regional services.

- 1944-1989 – Private veterinary practice during the period of central-planned economy in Bulgaria.

Act of the veterinary activity (1967) – veterinary activity is permitted only in structures subordinate to the Ministry of agriculture and in this way having a private veterinary practice become impossible.

- 1990-2009 – Private veterinary practice in conditions of passage to market economy and eurointegration.

Act of changes and additions to the Act of the veterinary activity (1992), Act of the veterinary activities (1999), Act of the veterinary activities (2006) – officially private practice is permitted to qualified veterinary specialists (including technicians under control of veterinary surgeon).

Act of the professional organization of the veterinary surgeons in Bulgaria (2008) – veterinary surgeons could practice on their own after 3 years of professional training.

The formation of these four conventional periods is based on the enacted and operative at each period normative acts, which regulated the rules for private practice. The common results from the study of these periods are given in table 1, where the chances for free practice of the profession are clearly stated.

Table 1.

Development of the veterinary medicine and private practice in Bulgaria

Development of the veterinary medicine	Act, regulating the veterinary profession	Private practice
I period /1878-1924/	1889 – Sanitary Act 1898 – Act of the sanitary-veterinary police 1906 – Act of the sanitary-veterinary service and police	**Permitted**
II period /1924-1944/	1924 – Act of the sanitary-veterinary service	**Permitted**
III period /1944-1989/	1967 – Act of the veterinary activity	**Not permitted**
IV period /1990-2009/	1992 – Act of changes and additions to the Act of veterinary activity 1999 – Act of the veterinary activities 2006 – Act of the veterinary activities 2008 – Act of the professional organization of the veterinary surgeons	**Permitted**

At the present stage of development of the society the market economy offers great opportunities for establishment of a private practice. After having a registration at the Regional Veterinary Service

(RVS) and the Regional Body of the Professional Organization (RBPO), every veterinary surgeon with three-year professional experience has the right to found his/her own clinic, dispensary or laboratory. At the same time the veterinarian has the chance to practise at local veterinary unit and perform the measures, planned in the State Preventive Programme (SPP).

The veterinary practitioners are charged by the operative in the last three years acts, to perform completely the treatment and the preventive activities. The veterinary specialists in the state services are engaged only with control functions.

On the following diagram (fig. 1) the proportion between the private veterinarians and the veterinarians on state posts, is given. It is clearly shown that the part of the practitioners (42%) and that of the veterinary officers (32%) are approximately equal. This proportion soon must be changed in a way of decreasing the number of state veterinarians and increasing the number of the veterinarians in practice. This will better the serving of the animal husbandry in compliance with the strategy "Prevention is better than cure" of the World Animal Health Organization.

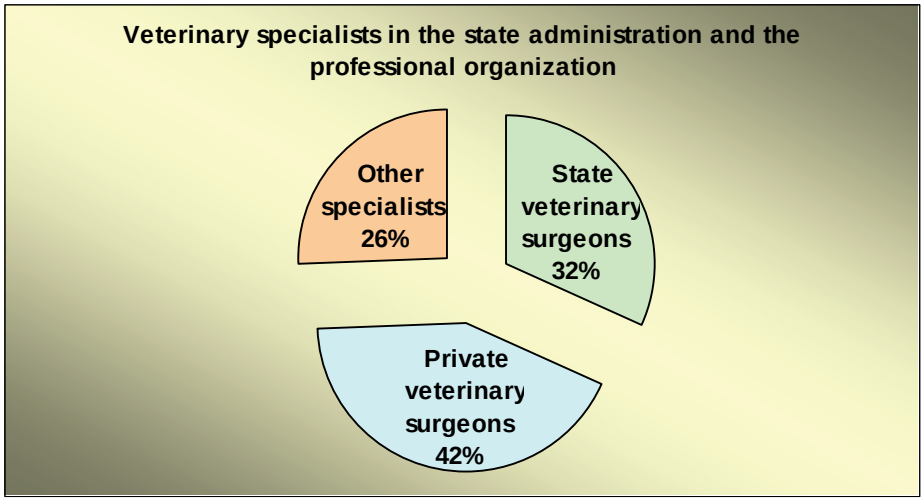

Fig. 1. Proportion between the veterinary practitioners and veterinary surgeons at the state services, 2009.

The legislation-based chances in the last years are supposed to provoke a great interest by the veterinary surgeons. At this time in the country there are 1559 registered private veterinary practitioners, who are very irregularly allocated due to the specific characteristics of every

administrative region. At fig. 2 the percentage of the private veterinarians based on their administrative registration, is given.

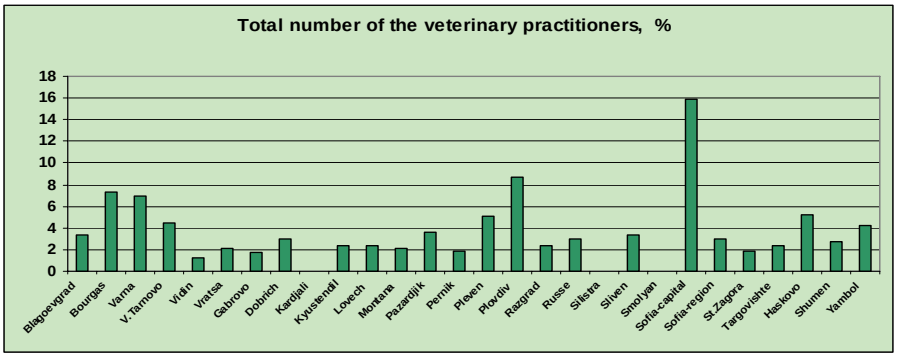

Fig. 2. Percentage of the veterinary surgeons with private practice by administrative division, 2009.

The figure above clearly shows that the capital and the other big cities which are economic centres of their administrative regions (Sofia, Plovdiv, Bourgas, Varna), take higher relative part of the private veterinarians. It could be said that the number of the veterinary surgeons with private practice there corresponds with the big number of citizens, respectively potential clients and owners of patients for veterinary practices. At the same time the more intensive development of the economy in these cities appears a significant stimulus for establishment of a private practice.

After dividing the veterinarians registered for private practice to women and men, a very huge difference in the proportion is noticed. The results are given in fig. 3.

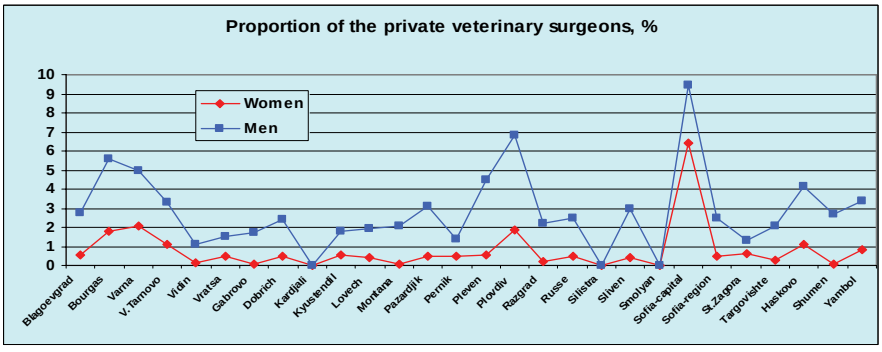

Fig. 3. Proportion women:men for the registered private veterinary practitioners by administrative regions, 2009.

In correspondence with the tendency that more private veterinarians practise in the big cities, the proportion above also shows that the relative part of women and men-veterinary surgeons in these cities is higher. Beside that it is obviously that the percentage of men significantly exceeds the percentage of women for every administrative region. As the National Veterinary Service (NVS) has contracted with 1040 veterinarians for executing the measures in the SPP, it is supposed that the main part of these veterinarians are men because of the significant physical labour. The rest 519 registered veterinary surgeons who are not in charge at the local veterinary units, have the chance to practise in clinic, dispensary or laboratory. This is preferred by the women-veterinary surgeons as a way of professional realization because of the more flexible working time, less physical labour and more opportunities for specialization.

The results from studying the women:men proportion of the veterinary surgeons with private practice, show that the tendency of feminization of the profession has not reflected yet on the practice as it has on the sphere of education for example. Women are only 21,91% from the total umber of the registered veterinary practitioners vs. 78,09% men. For comparison women present 66,25% of the admitted students in veterinary medicine in 2008 (Faculty of Veterinary Medicine, Trakia University, Stara Zagora) while men are just 33,75% (own studies, 2008).

CONCLUSIONS

The study of the veterinary legislation, regulating the veterinary activities and the present state of the private veterinary practice, allows us to make some conclusions:

1. The development of the private veterinary practice in Bulgaria passed in conformity with the social, economic and political conditions of the country.
2. During the period of central planning (1944-1989) in Bulgaria was not permitted for veterinarians to have private practice.
3. With an Act of the Parliament in 1992 after the democratic changes the private veterinary practice was legally permitted.
4. The allocation of the veterinary practitioners in the country is irregular because of the economic and social conditions, different for every administrative region.

5. The tendency of feminization of the profession has not reflected yet on the private practice – more than 78% of the practitioners are men.

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TEMPORAL ESTROUS SYNCHRONIZING BETWEEN DONOR AND RECIPIENT COWS

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Key words: poliovulation, embryos, synchronization, cow, protocol

SUMMARY

In this study we revealed the results obtained after we applied 4 protocols of poliovulation and estrus synchronization, in batches being formed each one of 15 donor cows and 64 recipients (a total of 60 donor cows and 256 recipients), from Montbeliarde and Prime Holstein breed.
We tried to establish estrus synchronization between donor and recipient in terms of time, females were observed to detected the heats, occurring as a consequence of the 4 poliovulation protocols. Recipients presented estrus with 18.04 hours before the donors, with variability between 6 and 44 hours for Protocol 1.
The estrus occurred, in protocol 2, at 17.96 hours, with variability between 3 and 32 hours.
In protocol 3, estrus occurred, on average, at 11.51 hours with a variation from 2-31 hours.
Protocol 4 has the effect of estrus emergence on average at 13.44 hours, with variations between 3-33 hours.
The best synchronization was obtained in protocol 3, and the timing was poor in protocol 1 (11.51 hours versus 18.04 hours).

1. MATERIAL AND METHOD

Researches were conducted in the period 15.10.2005 - 30.06.2009 in three farms in Southeastern Romania, on cows from Montbeliarde and Holstein breed.

For the 4 protocols of poliovulation a total of 60 donors and 256 recipient were used. For each protocol one batch with 15 donors and 64 recipients, both of Montbeliarde and the Prime Holstein breed was used.

Artificial insemination was performed with frozen semen from tested and selected bulls.

Each of the four groups was treated with one poliovulation protocol. In protocol 1, PMSG (Folligon, Intervet Holland) was used as stimulating hormone, Pridoestrol (vaginal spirals, CEVA - Santé) for blocking the sexual cycle, $PGF_2\alpha$ (Synchromate, Bremer Pharma - Germany) administered simultaneously (day 7-a) the two lots and Gn-RH (Receptal, Intervet - Netherlands) (Bîrțoiu, I.A. et all., 2007).

Protocol 1 of poliovulation started on day 9^{th} of the sexual cycle, which represented day 0 of the poliovulation protocol, Table 1.

Table 1.

Inducing the poliovulation in cows
using PMSG as follicular stimulating hormone (Protocol 1)

DAY	DONOR	RECIPIENT
0	**Introducing Pridoestrol intravaginal**	*Without treatment*
1	*Without treatment*	*Without treatment*
2	*Without treatment*	*Without treatment*
3	*Without treatment*	*Without treatment*
4	*Without treatment*	*Without treatment*
5	*Without treatment*	*Without treatment*
6	*Without treatment*	*Without treatment*
7	**IM 500 mcg $PgF_{2\alpha}$ a.m. 500 mcg $PgF_{2\alpha}$ p.m.**	**IM 500 mcg $PgF_{2\alpha}$ a.m. 500 mcg $PgF_{2\alpha}$ p.m**
8	*Without treatment*	*Without treatment*
9	**Extracting Pridoestrol IM 2000 UI PMSG**	*Without treatment*
10	*Without treatment*	*Without treatment*
11	**Estrus Gn-RH IM A.I. a.m. A.I. p.m.**	*Without treatment*
12	**A.I. a.m.**	*Without treatment*
13	*Without treatment*	*Without treatment*
14	*Without treatment*	*Without treatment*
15	*Without treatment*	*Without treatment*
16	*Without treatment*	*Without treatment*
17	*Without treatment*	*Without treatment*
18	*Without treatment*	*Without treatment*
19	**Embryo Recovery**	**Embryo Transfer**

Protocol 2 Folltropin (Bioniche Animal Helth-Ireland, highly purified extract, obtained from anterior pituitary from pig) used as stimulating hormone in days 4-7, Pridoestrol on day 0 and extracted on day 7 and GnRH on day 9 in donor and $PGF_2\alpha$ in the recipient (double treatment) 6^{th} day, by the schedule presented in Table 2 (Folman Y. et all., 1990).

Table 2.

Inducing the poliovulation in cows,
using FSH as follicular stimulating hormone (identical dosis), (Protocol 2)

DAY	DONOR	RECIPIENT
0	**Introducing Pridoestrol intravaginal**	*Without treatment*
1	*Without treatment*	*Without treatment*
2	*Without treatment*	*Without treatment*
3	*Without treatment*	*Without treatment*
4	**IM FSH (Folltropin) a.m. 2,5 ml (87,5 U.I.) p.m. 2,5 ml (87,5**	*Without treatment*

	U.I.)	
5	**IM FSH (Folltropin) a.m. 2,5 ml (87,5 U.I.)** **p.m. 2,5 ml (87,5 U.I.)**	*Without treatment*
6	**IM FSH (Folltropin) a.m. 2,5 ml (87,5 U.I.)** **p.m. 2,5 ml (87,5 U.I.)**	**IM a.m. 500 mcg $PgF_2\alpha$** **p.m. 500 mcg $PgF_2\alpha$**
7	**IM FSH (Folltropin) a.m. 2,5 ml (87,5 U.I.)** **p.m. 2,5 ml (87,5 U.I.)** **IM $PgF_2\alpha$ a.m. 2 ml (500 mcg)** **p.m. 2 ml (500 mcg)** **Extracting PRIDOESTROL p.m.**	*Without treatment*
8	*Without treatment*	*Without treatment*
9	**ESTRU** **IM Gn-RH** **A.I. a.m.** **A.I. p.m.**	*Without treatment*
10	**A.I. a.m.**	*Without treatment*
11	*Without treatment*	*Without treatment*
12	*Without treatment*	*Without treatment*
13	*Without treatment*	*Without treatment*
14	*Without treatment*	*Without treatment*
15	*Without treatment*	*Without treatment*
16	**Embryo Recovery**	**Embryo Transfer**

In protocol 3 Folltropin in decreasing doses (days 4 to 7) was used as stimulating hormone following the Pridoestrol (day 0 to 7) and GnRH, on 9th day. The recipient was given 6 $PGF_2\alpha$ twice on day, Table 3 (Seidel, GE, Jr., Seidel, MS, 1991).

Table 3.

Inducing the poliovulation in cows,
using FSH as follicular stimulating hormone (decreasing dosis), (Protocol 3)

DAY	DONOR	RECIPIENT
0	**Introducing Pridoestrol intravaginal**	*Without treatment*
1	*Without treatment*	*Without treatment*
2	*Without treatment*	*Without treatment*
3	*Without treatment*	*Without treatment*
4	**IM FSH (Folltropin) a.m. 4 ml (140 U.I.)** **p.m. 4 ml (140 U.I.)**	*Without treatment*
5	**IM FSH (Folltropin) a.m. 3 ml (105 U.I.)** **p.m. 3 ml (105 U.I.)**	*Without treatment*
6	**IM FSH (Folltropin) a.m. 2 ml (70 U.I.)** **p.m. 2 ml (70 U.I.)**	**IM a.m. 500 mcg $PgF_2\alpha$** **p.m. 500 mcg**

		$PgF_2\alpha$
7	**IM FSH (Folltropin) a.m. 1 ml (35 U.I.)** **p.m. 1 ml (35 U.I.)** **IM $PgF_2\alpha$ a.m. 2 ml (500 mcg)** **p.m. 2 ml (500 mcg)** **Extracting PRIDOESTROL p.m.**	*Without treatment*
8	*Without treatment*	*Without treatment*
9	**Estrus** **Gn-RH IM** **A.I. a.m.** **A.I. p.m.**	*Without treatment*
10	**A.I. a.m.**	*Without treatment*
11	*Without treatment*	*Without treatment*
12	*Without treatment*	*Without treatment*
13	*Without treatment*	*Without treatment*
14	*Without treatment*	*Without treatment*
15	*Without treatment*	*Without treatment*
16	**Embryo Recovery**	**Embryo Transfer**

In protocol 4 FSH combined with LH (FSH Pluset, the company Sykes Vet International)was used for follicular stimulation. The product was administered 5 days (2 daily applications) (Pursley, JR et all, 1995) on days 0-4, in decreasing doses. On the 5th day, $PGF_2\alpha$ was inoculated (2 times) in donors. Recipients have received two doses of $PGF_2\alpha$ on day 1, Table 4.

Table 4.

Inducing the poliovulation in cows, using FSH as follicular stimulating hormone + LH, into an commercial product (Protocol 4)

DAY	DONOR	RECIPIENT
0	**08:00 - 3.0 ml IM (150 IU FSH + 150 IU LH)** **20:00 - 3.0 ml IM (150 IU FSH + 150 IU LH)** Day 11 of the sexual cycle	*Without treatment*
1	**08:00 - 2.5 ml IM (125 IU FSH + 125 IU LH)** **20:00 - 2.5 ml IM (125 IU FSH + 125 IU LH)**	**IM $PgF_2\alpha$** **a.m. 2 ml (500 mcg)** **p.m. 2 ml (500 mcg)**
2	**08:00 - 2.0 ml IM (100 IU FSH + 100 IU LH)** **20:00 - 2.0 ml IM (100 IU FSH + 100 IU LH)** **IM $PgF_2\alpha$ a.m. 2 ml (500 mcg)** **p.m. 2 ml (500 mcg)**	
3	**08:00 - 1.5 ml IM (75 IU FSH + 75 IU LH)** **20:00 - 1.5 ml IM (75 IU FSH + 75 IU LH)**	*Without treatment*
4	**08:00 - 1.0 ml IM (50 IU FSH + 50 IU LH)** **20:00 - 1.0 ml IM (50 IU FSH + 50 IU LH)**	*Without treatment*
5	**Estrus**	*Without treatment*

	Gn-RH IM A.I. a.m A.I.. p.m.	
6	A.I. a.m.	*Without treatment*
7	*Without treatment*	*Without treatment*
8	*Without treatment*	*Without treatment*
9	*Without treatment*	*Without treatment*
10	*Without treatment*	*Without treatment*
11	*Without treatment*	*Without treatment*
12	**Embryo Recovery**	**Embryo Transfer**

There have been followed both donors and recipients subject to the 4 protocols poliovulation. Females were isolated from the rest of the cows, separated donors in boxes in maternity and recipients are best left to the special pens in loose housing, with food *ad libitum*.

Knowing that is very important to get the best possible synchronization between donor and recipient, to obtain the best possible rate of pregnancy, females were followed both donors and recipients, and the exact day and hour when they had estrus were recorded (Păcală N., 2004).

The cows were followed carefully for clinical signs of estrus, the specific mucus, jumping on other females or increased locomotion activity (Robertson E., 2005).

Recipient females that showed estrus at least 24 hours before the female donor and at most 12 hours after female donor estrus were accepted for transfer.

Once recorded the times when females showed the first signs of oestrus, the results were recorded.

2. RESULTS AND DISCUTIONS

Following application of the ***protocol 1 of poliovulation*** in 15 donor cows and 64 recipient cows the following results were obtained.

Recipient cows showed estrus 18.04 hours on average before donor cows. This is explained by the fact that donors showed estrus later than the other protocols, respectively 72 hours after $PgF_2\alpha$ administration, leading to the asynchronous between donor and recipient females.

The best synchronization was of 6 hours between the donor cow and the recipient cow and the worst was 44 hours, between donor with number 6799 and recipient with number 6643.

Following application of ***protocol 2 of poliovulation*** in 15 donor cows and 64 recipient cows were obtained the following results:

Recipient cows showed oestrus on average with 17.96 hours before the donor cows.

Although we got a better synchronization between donor and recipient females, however, in this case there is also a relatively large distance, on average, between recipients and donors estrus. This is because of FSH doses administered evenly throughout the poliovulation protocol.

The best synchronization was of 6 hours between the donor cow with registration number 1563 and the recipient cow with registration number 2287 and the worst synchronization was of 33 hours, between donor cow with registration number 7437 and the recipient cow with registration number 4845.

It is noted that the distance between donors and recipients estrus is not so high and it is reduced at maximum 33 hours in comparison with 44 hours, maximum obtained in poliovulation protocol 1. Also, the best synchronization decreases from 6 hours in poliovulation protocol 1, to only 3 hours, in poliovulation protocol 2.

Following implementation of ***protocol 3 of poliovulation*** to 15 donor cows and 64 recipient cows the following results were obtained:

Recipient cows showed estrus at 11.51 hours before the donor, on average.

Using this protocol, we get a much better synchronization between donor and recipient females. This is due to the administration of FSH in decreasing doses.

The best synchronization was of 2 hours between the donor cow with registration number 2881 and the recipient cow with registration number 4803 and between the donor cow with registration number 3081 and the recipient cow with registration number 4995 and the worst synchronization was of 32 hours, between donor cow with registration number 2070 and the recipient cow with registration number 4891.

It is noted that the distance between donors and recipients estrus it is reduced within 32 hours, lower than the first two poliovulation protocols. Also, the best timing is just about 2 hours in protocol 3 of poliovulation, in comparison with 6 respectively 3 hours in the first two poliovulation protocols.

Following realisation of ***protocol 4 of poliovulation*** on the 15 donor cows and 64 recipient cows the following results were obtained:

Recipient cows showed estrus at 13.44 hours before the donors, on average.

Using this protocol, we obtained a relatively good synchronization between donor and recipient females. This is due to the good balance of

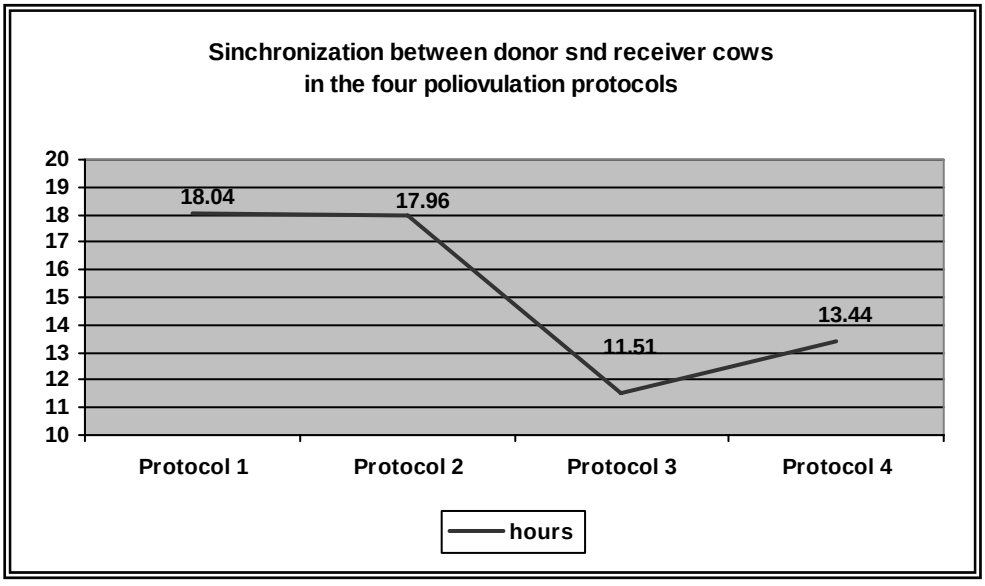
FSH and LH and to the ovulation within 60 hours after $PGF_2\alpha$ administration.

The best synchronization was of 3 hours between donor and recipient cows and the worst timing was 33 hours, between donor with registration number 2263 and recipient with registration number 4840.

It is noted that, although the maximum distance between donors and recipients estrus is still 33 hours and the best timing is 3 hours after protocol 4 of poliovulation, similar to those obtained in protocol 2 of poliovulation, the average obtained in protocol 4 of poliovulation is much better, ie 13.44 hours instead 17.96 hours following protocol 2 of poliovulation.

Analyzing the above data it is noted that, of the four protocols used, the best synchronization was achieved at protocol 3 of poliovulation, in which FSH was used as follicle stimulating hormone (in decreasing doses).

Graphic 1.



This is explained by the reaction of donors, who had presented estrus 48 hours after $PgF_2\alpha$ administration, leading to a better synchronization between donor and recipient females.

During protocol 3 of poliovulation, recipients showed estrus, on average, at 11.51 hours before the donors.

The worst synchronization was achieved at protocol 1 of poliovulation respectively to 18.04 hours before the donor. This is explained by the fact that donors showed estrus later than during the other protocols, respectively 72 hours after $PgF_2\alpha$ administration, leading to the asynchronous between donor and recipient females.

The best synchronization was achieved within protocol 3 of poliovulation, between the female donor 3081 and recipient 4995, respectively recipient presented estrus 2 hours before the donor. The same case in also met in the female donor 2881 and recipient 4803.

3. CONCLUSIONS

1. For better synchronization of estrus in females subjected to embryotransfer a pelvic examination should be performed before implementing protocols to identify if their sexual activity is cyclical and if at the examination of the ovaries, performed by transrectal and ultrasound examination, one of the ovaries has a yellow body and the other ovary is functioning well (cyclic ovarian activity) and if the uterus is well structured.

2. For a successful embryo transfer in cows it is necessary for receivers to be synchronized with donors as good as it is possible. It is preferred that receivers present estrus in the same time with donors or at most at 24 hours before them.

3. The best results were obtained by applying protocol 3 of poliovulation, which consisted in administration of FSH in decreasing doses, leading to a better structuring of the follicles and ovulation grouped much more uniform, which is expressed clinically by occurrence of estrus within 48 hours after $PGF_2\alpha$ administration.

4. For better synchronization of receivers with donors, $PGF_2\alpha$ should be given to receivers with more than 24 hours before administrating this drug to the donor.

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A SURVEY ARTICLE ON REPRODUCTIVE CYCLE AND MATING PATTERN OF *URSUS ARCTOS*

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Key words: bear, promiscuity, embryonic diapause, pseudopregnancy.

SUMMARY

The brown bear shows specific physiologic and behaviour characteristics during the reproductive period. Female exhibit a seasonal poliestrus cyclicity during which she mates promiscuously. The act of copulation triggers an induced ovulation, and once the fertilisation is made, the embryo enters into a slow developing phase called embryonic diapuase. With the mother's winter sleep the embryo finally attaches to the uterus, entering in a normal gestation development. Due to complete fasting and limited resources the mother gives birth to small cubs after a short post-implatation period (≈ two months). To assure better cub survival rate, the female protects them for a long period (up to 3.5 years), time in which she will show no estrus. By practicing sexually selected infanticide males are able to shorten the time to the female's next estrus. This paper summaries data already published and present an overview of brown bear's reproduction.

This paper tries to gather and summarise data already published and present an overview of the complex and particular physiology of brown bear's (*Ursus arctos*) reproduction. Characterization of brown bear's reproductive cycle provides a context in which to understand the endogenous rhythm of the gonadal function, which in turn determines the seasonal timing, physiologic peculiarity and behaviour during oestrus and birth.

Reproducting seasonality in ursides

In most animal species that have a distinctly seasonal pattern of mating, both sexes undergo annual variations in gonadal function [13]. For many of these seasonal animals, an endogenous circannual rhythm dictates the course and duration of the gonadal cycle [2,13]. Increased androgenic and oestrogenic steroidogenesis in both sexes begins approximately in March in the seasonal Ursini [39,55,56,65,75,84,85,108,109], which corresponds to shortly after the spring equinox when day length exceeds 12 hours. The circannual testicular cycle is appropriately phase-synchronized with the timing of oestrus in females. The stage of peak testicular function generally overlaps the temporal distribution of oestrus for each of the ursid

species [90]. Photoperiod is likely to be the principal zeitgeber for the endogenous circannual gonadal rhythms in ursides [90].

Increasingly seasonal climatic conditions during the Pleistocene [14,37,91] acted as the ultimate evolutionary pressures to the development of the brown bears obligate mode of seasonality [90]. Phylogenetic inertia explains at least 83% of the birth and oestrus timing in ursids, strongly indicating that seasonality is an evolved trait in the ursids [90].

Spady *et al.* (2007) concludes that the lack of latitudinal variance in the timing of birth and oestrus among the Temperate species of bear, in combination with the distinctly seasonal distribution of oestrus and birth, provides strong evidence that ursids exhibit the trait of reproductive seasonality [90].

Within the Temperate Zone, *Ursus arctos*, has mating season of approximately two to tree months, from late spring to early summer (late May/early July) [3,4,36,50,73,90,116]. The duration of oestrus is closely associated to phylogeny, with long upper-range limits (9–19 days), and a mean oestrus length of 4 days [90]. The longer oestrus provides the female greater temporal flexibility in finding a mate before ovulation [90].

Breading pattern

Bears usually exhibit sex-biased natal dispersal: females are highly philopatric, establishing their breeding home ranges in or near their natal areas, whereas males disperse from their mothers home range and can move long distances [10,42,45,64,78,87]. They both show having intra- and inter-sexually overlapping ranges [6,22,76], roaming widely to mate and decrease their range after the mating season [24].

According to Kordek and Lindzey (1980), 88 % of the females will breed by the time they reach 3.5 years of age [60]. Brown bears are sexually dimorphic [53] with both sexes mating promiscuously (cases of females mating with up to eight males were reported) [6,7,8,19,76,88,100]. Data suggest that female bears neither prefer genetically distant nor close males [7]. The couple remains together for a period that ranges from few hours to several days [7]. Some males copulate and leave the female shortly thereafter and some may consort for up to 2 weeks [19,52].

In brief, bears undergo a breeding pattern similar to many other mammalian species (e.g. cattle, sheep, cats), which can be divided into three standard segments: courtship, mating, and refractory period. Courtship begins with an obvious movement by the male toward the

female and ends when the male mounts the female. It includes posturing by the male, urogenital sniffing of the female, as well as auditory emissions to the female. If the female is responsive, she will undergo lordosis, and subsequently will be mounted by the male [12].

Mating includes mounting and pelvic thrusts that are often followed by a flutter. The refractory period begins with a dismount by the male or a ‘‘roll-out’’ by the female to break the union. During the refractory period, the male is no longer interested in the female and the female is less receptive to a male [12].

Even if females do not choose their mate, they may still have the post mating opportunity to choose between the sperm of several males (cryptic female choice [31]) by direct comparison [7]. Either the most heterozygous sperm outcompete the rest by being the fittest [40], or a female is able to evaluate male sperm quality and select the most heterozygous sperm [9].

Polyoestrus cyclicity

Ursids have traditionally been classified as mono-oestrous species [15,29,51,94], and earlier observations to the contrary [77,92,112] were discounted as artifacts of captivity, but evidence for seasonal polyoestrous cyclicity in wild populations of ursids is mounting [19,20,67,68,93].

Craighead *et al*. (1995) proposed that the multiple distinct oestruses observed in wild *U. arctos* are the result of discrete, successive waves of follicular development, and that the interval between coitus represents the time needed for the subsequent wave of follicles to mature following ovulation of the previous wave [19]. Each corpus luteum becomes dormant following ovulation, allowing females to re-enter oestrus after conception. Due to this fact multiple paternity of litters has been documented by genetic studies in wild *U. arctos* [21]. Data showed that 51% of wild brown bear females which formed male–female pairs during the mating season, did so on two or three separate occasions in that season, suggesting that re-entry into oestrus is a common reproductive strategy for ursids in the wild [93].

Induced ovulation

Spontaneous ovulation is a process in which ovulation occurs as a result of a hormonal sequence of events at a specific time in the reproductive cycle and is independent of mating [83]. In induced ovulation, the act of copulation initiates a series of neural events that pass to the brain and lead to the release of one or more oocytes.

Using laparoscopy and radioimmunoassay, Boone et al. (2004) demonstrated that most American black bears are induced ovulators (those findings were also confirmed by observing the number of corpus leuteum). It would appear that, like most cats, bears require vaginal touch to induce the ovulation. Another observation shows that few bears are also capable to release ova without neurological response provided by the mating process [12].

Progesteron levels

Study on the Asiatic black bear showed that progesterone concentration of a pregnant female may rise gradually in the months after mating, then increase sharply near the time of implantation, before returning to basal levels near parturition [84]. The same pattern was seen for the brown bear where progesterone concentration remained near baseline among nonmated females (0.32–1.50 ng/ml), and increased among mated females (0.70–2.70 ng/ml) as post-mating interval increased. Others report similar baseline values from the serum taken 210–300 days pre-parturition (0.4–1.1 ng/ml)[86,107].

Fecal progesterone concentration is also considered to be appropriate for monitoring luteal activity in the brown bear [27,43]. Like serum values, fecal porgesterone concentration were reported to be low during the pre-mating period (0.98 + 2.04 ng/g feces) and tended to be elevated during the post-mating period (7.36 + 7.94 ng/g feces [27].

Embryonic diapause

From the moment of ovulation to peri-implantation, the corpus luteum remains relatively dormant, period known as the corpus luteum dormancy phase [32,107]. This period, started with the fertilisation of the ova, is a peculiar evolutionary adaptation known as delayed implantation. The embryo develops to the blastocyst stage, then enters a period of embryonic diapause [28,47,98,107,110,116], during which the blastocyst remains unattached in the uterus [26]. The dormant condition is characterized by a very low mitotic index and reduced metabolic activity [26,46], although some growth is apparent [116].

Pathological exams made on black bears by Kordek and Lindzey (1980) showed that all blastocysts were free-floating within the lumen or were unattached within the rugose folds of the uterus. The inner cell mass and trophoblast were evident and blastocysts were enclosed in a zona pellucida, as it is described by Wimsatt (1963) [60].

Delayed Implantation and the active luteal phase

Implantation of the embryos is delayed until November [80,90] when the female retires to her winter den [3,50,116]. Because of embryonic diapause, the cumulative embryos all implant at the same time and comprise a single litter [90]. Coincident with implantation, there is a marked elevation in plasma progesterone levels in late November/early December [36]. This sharp rise in progesterone (4650 ng/g -[27]) is returning to basal levels near parturition [36,49,75,85,107,108].

Like the dormancy phase of the corpus luteum, the active luteal phase in brown bear is generally of shorter duration (~ 3 months on average) [90]. Reactivation of corpora lutea is made by unidentified hormonal triggers that initiate the prolonged luteal phase of the reproductive cycle [32,36,49,84,85,86,107,110,116,117] and roughly corresponds to shortly after the autumn equinox when day length is less than 12 hours and decreasing most precipitously [90]. The established mechanism for photoperiod stimulation of corpus luteum reactivation, and consequent implantation, involves a neurotransmitter-hormonal cascade along the pineal–hypothalamic–pituitary–gonadal axis triggered by retinal (and perhaps extra-optic) perception of environmental photoperiod cues [16,66].

Comparison of luteal tissue of pre-implantation animals (June-September) with that of post-implantation specimens (December) clearly showed a marked alteration in luteal morphology, with a two to four fold increase in luteal volume by December [116,60]. The sources of ova were found to be equally divided between ovaries and the heaviest ovaries were those bearing corpora lutea, with each corpus adding about 0.50-1.5 g to ovarian weight [60].

Parturition

The temporal distribution of births varies little by latitude among the five species of bear that reside primarily within the Arctic or Temperate Zone and is more seasonally restricted than that of oestrus [90].

After six to eight weeks of gestation, while still hibernating in dens, the female brown bears gives birth in late January to one to four altricial small cubs (weighing only 250-400g) [36,76,107,116,]. The period of lactation is on the other hand relatively long, extending at least for four to five months [54]. This prevents the entrance in the next oestrous cycle, which will make breeding occur only every other year [7,23,32,79].

One of the most striking observations is that the female bears give birth to the cubs wile fasting completely [54]. It has to be noted that pregnancy and lactation is absolutely impossible for other carnivores under starvation [71].

The female brown bear must make it through winter with the help of fat stores and she must also provide milk for her cubs. Because the bear does not drink nor eat during winter sleep, it must be extremely careful with its utilization of water and amino acids. For those reasons the volume of the uterus (liquid volume) cannot grow too large, which would be necessary in the case of an efficient protein synthesis. On the other hand, procuring the amino acids needed for protein synthesis is auto-limiting despite the fact that novo synthesis for essential amino acids was suggested. Fetus needs lots of carbohydrates, mainly glucose, for its development. A female bear cannot, however, store large amounts of carbohydrates in her body, she must in fact manufacture them during winter sleep, mainly from amino acids. Therefore, from a developmental point of view it is advantageous for a cub to switch over to milk as early as possible [54]. Milk contains everything a cub needs for its development (milk fat level of the grizzly female bear in the middle of lactation is 19% [41,59,72]), while the carbohydrate concentration is 1-3% [34]. The short period of post-implantation gestation, limits the energetic cost of reproduction by truncating embryonic development, which in turn reduces the size of offspring and thereby reduces the initial costs of lactation [90].

In the case of primiparous females, they give birth to significantly smaller litters of cubs and also have a higher probability of cub loss than multiparous mothers [118]. Lack of behavioural skills associated with foraging and parental care [5,113] may force younger females to make a relatively greater reproductive effort than fully grown females [18,35]. Larger size with age may improve the status of subdominant females, which probably increases the possibility for successfully rearing offspring [97].

Pseudopregnancy

Pseudopregnancy is the result of obligate seasonal reactivation and maintenance of the corpus luteum in the absence of an embryo (duration equivalent to that necessary for normal gestation)[70]. The absence of maternal-embryonic contact during the lengthy delayed implantation period is a barrier to maternal recognition of pregnancy. Therefore, Schulz et al. (2003) hypothesize that black bears maintain corpus

luteum, even in the absence of embryos [86], phenomenon consistent with pseudopregnancy.

Non-mated brown bears have also been documented to experience pseudopregnancy [107]. It was seen that progesterone profiles are indistinguishable from those of pregnancy, making pregnancy diagnosis extremely difficult [49,85,86,106,107]. Slightly higher progesterone levels were also observed in lactational anoestrus (range = 1,7-2,5 ng/ml) as compared to nonlactational anoestrus (range = 0,6-1,4 ng/ml), this difference is not felt to be significant [21].

On the other hand the existence of pseudopregnancy could facilitate embryonic transfer in bears. Treatment of recipients with hCG prior to embryo transfer could induce ovulation and establish a corpus luteum capable of producing progesterone for the length of normal pregnancy [36].

Weaning

Brown bear offspring separate from their mothers after a long period of infant dependency, (1.4–3.5 years old) [24,48,63,] and were documented of being weaned with body masses varying from 17 to 69 kg [25]. While caring for her young, the female will not mate until she separates completely from them, which in nearly all cases coincides with the mating season of late May/early July [99,25]. Nursing of young >1 year of age has a positive effect on their growth rate but is energetically costly for mothers and reduces the number of litters a female can produce during her lifetime [7,24]. This could explain why in southern Sweden and in populations elsewhere in Europe, most brown bears are weaned as yearlings [38,99].

A general trend in the age of weaning seems to be that offspring are weaned when they reach a threshold size [61]. Hence the length of maternal investment often varies in relation to nutritional and maternal condition [17,33,104] this period is flexible for each litter size [22].

In the family breakup reported by Herrero and Hamer (1977), yearlings made several attempts to reunite with the mother by approaching her and the adult male, but each time they ran away [52]. Rogers (1987) found that adult females recognize their weaned offspring and tolerate them in their territories [81]. In addition, adult females actively aided their daughters in establishing territories by shifting their area of use away from the daughter as she approached maturity These results indicate that females generally are located geographically close to their relatives, whereas males are located at random compared to their

relatives. Although not gregarious, *U. arctos* may be more social than previously assumed [96].

SSI and female counterstrategies for litter survival

Infanticide is an adaptive behavioral strategy to increase the fitness of the perpetrator [111]. Sexually selected infanticide (SSI) by brown bear males [58] is a major cause of bear cub mortality (averages 35% annually cub deaths in the southern Sweden) [19,69,101,102,103,105,118]. Losing an unweaned cub shortens the time to the mother's next estrus [7,101], which will become receptive again within two to four days [7]. By doing this, the male perpetrator increases his own opportunity to breed, which gives him a higher probability of siring the female's next litter [58].

To protect the litter, females may have to benefit form the large ranges during mating season through mate selection by male–male competition or female choice [1], hiding paternity as a counterstrategy against infanticide [30], fertilization insurance [44], sperm competition [95], and selection of the most genetically compatible sperm [115]. By being promiscuous, females might mate with the geographically closest partners [58] and select a father for their offspring via post-copulatory cryptic choice. This behaviour may confuse paternity assessment by males, which tend not to kill infants of females they have copulated with (57,89). Females with cubs also choose sexual segregation in habitat, [74,25,114] minimizing their range size during the mating season [11,62,82].

CONCLUSION

One of the greatest threats to the conservation of the brown bear is the alteration and loss of habitat, caused directly or indirectly by humans. Human encroachment on wild bear habitat has undoubtedly altered bear's choice of terrain and home range sizes. Adding to this, bear's wide roaming range during the mating period and the maternal aggressiveness shown by females with cubs, makes human-bear encounter and with this, accidents inevitable. A better understanding of the reproductive physiological factors that influence the bear's behavior during mating and maternal care period, will provide assistance in better wildlife management.

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RESARCH AND OBSERVATION ABOUT CALLUS BONE FORMATION AFTER ELONGATION IN SHEEP

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Key words: osteogenesis remodelation ,bone elongation,callus formation.

SUMARRY

A study has been effectuated about the manner of bone callus formation and remodelation, after repeated elongations.
For this purpose 12 sheep were used adulthood who performed at the mandibular symphysis osteotomy.After that sectioned fragments were then fixed to the jaw by a 2 branches elongated conical screws with 4 screws. Elongarea was performed daily 0.5 mm.Remodelarea mandibular bone between the two areas began through a primary callus which gradually ossified. Primary callus has gone since week 4 to week 8 after completion lungimii.The healing of the bone was observed by clinical examination and X-ray examination carried out successively for 12 weeks.

Callus bone formation is a complex process that begins immediately after the fracture and lasts a variable time depending on many factors.The prosess of fracture healing was studied on different animals in different types of fractures and depending on the purpose of studies. Immobilization or fixation of the fracture site determines the level of training gag cartilage formation and healing way. It is generally accepted that the osteotomy metafizar induces a strong osteogenic response in bone lengthening osteotomy than diafizar. It is also known to use an oscillating saw (Frierosn and col.) Induces remodeling osteotomy for late consolidation interfragmentare portion. Also, change and adaptation elongation rate of over 2 mm / day results in moderate degeneration (Shujuan Zou, 2001) of articular cartilage.

1.MATERIAL AND METHOD

The motivation for this study starts from the need to correct some states maloclusions arising from animals, most often congenital or acquired in cases of accidents or fractures consolidated vicious.

Malocluzia In such situations becomes a disturbing factor in terms of physical function, muscle, ligament, etc.. are also encountered situations bulging asymmetric in road accidents lack of substance in the reconstruction of bone segments must be in good condition for a normal and proper occlusion.

Experimental study was conducted at the Faculty of Veterinary Medicine Cluj-Napoca during 2007-2009, a total of 12 pan-bred sheep, aged 2-5 years, gender female, with body weight between 25-35 kg . Before surgery the sheep were anesthetized with xylazine generally 0.3 mg / kg iv and ketamine 10 mg / kg iv, prepared locally by clipping, shaving, disinfection and local anesthesia with epinephrine xilină + 2% in the amount of 6 ml.

The place of choice was the middle (junction) of mandibular symphysis jaw (fig. 1) by mucosal incision and periosteal dilacerarea (2ig.2). With a specific milling was performed osteotomies corticotomia (modeling cortical orthodontic purposes) complete bone segment (fig. 3).

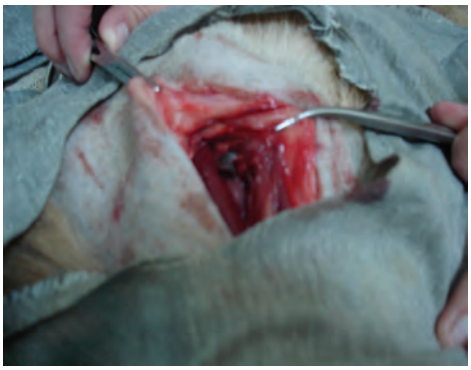

Fig.1. Skin and mucosal incizion

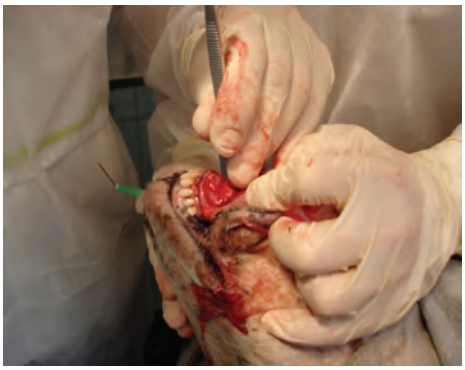

Fig.2. Periostal dilacerea

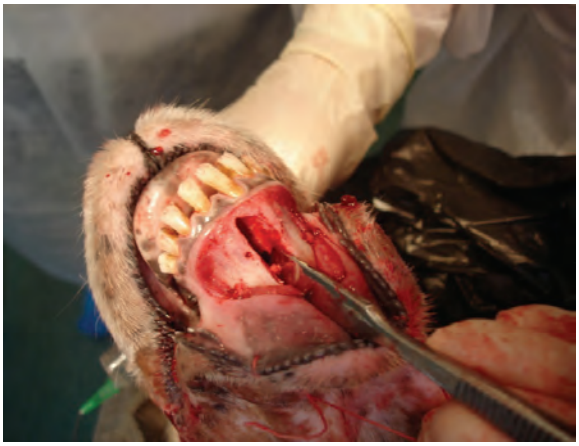

Fig.3. Osteotomy

After an elongated hemostasis (osteodistractor) made to order (which may remove bone fragments on the desired length) was fixed by

4 screws, conical screws on the mandibular second arms so as to give good stability (fig. 4). He then checked the stability and local haemostasis after the oral mucosa was sutured so as to cover as much elongatorul for it to not withhold food (fig.5).

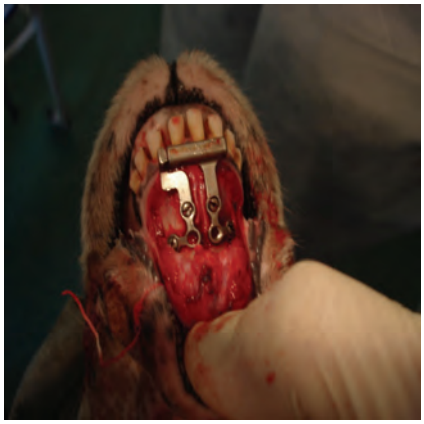

Fig.4.Elongator fixation

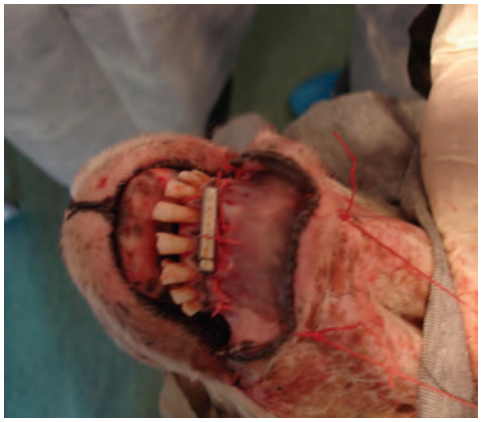

Fig.5. Opertative wound closure

From the second day after surgery was performed elongarea of 0.5 mm / day through a lever that is rotated under an angle. Elongation continued for 11 days. During labor the sheep are contention in quadrupedal position and mouth opening is easy. In the first 3-4 days after the operation we carried out local lavage (wash) with betadine ground. 1% ,osteodistractors processes (elongations) was maintained for 10 weeks.

2.RESULTS AND DISCUSSIONS

It is known that after the fracture, the cells in focal role of defense, responding promptly to messages quickly and efficiently, local and systemic stimuli and secondary biochemical and biophysical they issue local mediators to determine the response to aggression. This perception and biological awareness lasts by some authors (Frost, 1989, Greenbaum and Kant, 1993, Einhorn, 1998 Weiss 1999) up to 7 days.

The release of lysosomal enzymes, pH acidification local development and organization of macrophages, white blood cell development with other inflammatory cells occupying the interval between broken edges, soft primary gag is the level of training. During training the callus cells are stimulated and sensitized start to produce vessels neoformație, fibroblasts, intracellular content and supporting

cells that together form the granulation tissue of broken edges, and the clinical aspect coaptarea is determined by fibrous tissue in the form of callus string.

During cellular displacement which were held in the focus of fracture are subject to a direct stretch that we agreed on. After the first week of the appearance of a callus pieces showed fibrous hypertrophy in length and height for the week 4th and 8th elongation after stopping it to disappear gradually.

Other authors (Delloye C., et al, 1990; Komuro Y, et al., 1994; Korkut O., et al., 1988) notes gag fiber disappearance after 4 weeks of stopping displacement probably because lower period elongation rate of elongation also lower. Radiological examinations performed regularly at 7 days shows that the osteotomy is highlights from a densification tissue between the edges cut.

At 14 days of the osteotomy are highlights fiber contour gag slightly elongated round bone islands. All currently observed obvious periosteal reaction along the length of the fracture line. At 5 weeks dance is formed along the length incision, bone gag easily be observing both radiographically and by palpation in the area of impact. After 10 weeks elongatorul was built by moving the sinuses. Removal was made very easily and the holes present the screws were covered in a few days. Concluded that this method can be applied to any animal in a state of bulging accidental or induced or malocluzie disorders.

3.CONCLUSIONS

3.1. Experimental conditions elongarea bone in sheep in the rate and elongation rate, during osteotomies, waiting period, stability Catchers bone age of the animal, the method and type of food, feed quality, play an important role in ossification gradualitatea and dynamics of each stage.

3.2. The stability of the fracture line directly affects bone gag. The presence of even small mobility causes the formation of a callus higher.

3.3. The callus of elongated osteogenezic of our study was predominantly a composite intramembranoasă. Most new type of bone tissue was observed in the periosteum and Neocortex remodeling began at the end of the first week of the stretch and continued 21 weeks after its completion.

3.4. Callus bone formation in sheep after mandibular osteotomy performed at the level of normal over all sheep taken in observation and were followed for 12 weeks, the essential condition is the correct setting

and stabilization of fractured segments, the approach and content straight when manoperelor of elongation.

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MORPHOLOGICAL CHARACTERISTICS OF HORSE AND DONKEY EYE FUNDUS

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Key words: horse eye fundus, animal tapetum ,animal retina

SUMMARY

The morphologic and physiologic particularities of the eye fundus give him an important role in diagnostic of local and systemic diseases , because a big disturb of the body functions is perfect related at the eye fundus components .
Horses and donkeys too, can develop a variety of disease at the ocular level and sometimes are discovered too late or accidentally at a complete clinic exam .
A right diagnostic at the eye fundus level suppose a good knowledge of normal variations at the studied species.
The present paperwork proposes a horse and donkey eye fundus study with observations and notes the existent characteristics. We were interested the normal morphological aspect of every components of the posterior globe segment with appreciation of the form, color and space localization.
The ophthalmoscopyc exam was made at the Pathology Surgery Clinic of the Veterinary Medicine Faculty from Cluj.
The obtained results show that don't exist major morphological aspects between that two species from our study, the horse eye fundus is similar with the donkey eye fundus. Introductions
In the past , the ophthalmologic exam in horses consist by applications of different tests for visual testing when the horse is let free on a strange obstacle field .The identifications of ocular diseases in such appreciated species goes to implement of modern examination techniques that include obligatory eye fundus exam. Because in animals, eye fundus in particular to every species, in horses too exist some specie and age particularity's .
Used upon rarely, donkey, as a species, doesn't get the same attention in ophthalmology field as horses, and are a few dates about their eye fundus .
It is clear a thing: no matter of species, the research in ophthalmology field continues and the results are most over expectations.

1.MATERIAL AND METHOD

The study was made on a period of 16 months and included a number of 21 horses and 11 donkeys, healthy ophthalmological speaking, presented at the medical consult for different reasons: castration, plagues, and laminitis.

The examination was made in Surgery Clinic of Veterinary Medicine Faculty Cluj, and in stable too.

In our casuistry, from 21 horses,6 of them had the age until 1 year (we exam a foal with age 2 days).All the donkey taken for this study have been adults.

The indirect ophthalmoscopy technique was easy to do, we didn't find any difficulties, the exam was made with ought tranquilization, but for a good quality ne needed a dark room.

Indirect ophthalmoscopy technique

The principle of this method is the examination of animal ocular globe, an examination made with indirect ophthalmoscope (with light source and video camera incorporated) and with a lent between examiner (ophthalmoscope) and patient.The lent is not incorporated in ophthalmoscope so, in the time of examination she must be hold with a hand by the examiner. The lent dioptrically power is 20 D and we can obtain 4-5x magnification field of view. This lent must be settle at 4-5 cm from the patient eye and at 0,5 -.0,75 m from the examiner (this in an advantage for the examiner because he keep distances from the animal).

The obtained imagine by indirect ophthalmoscopy is real and upside down.In the present study, all patients have been exanimate with ought tranquilization, the contention was mad in a good and comfortable position for the animal and examiner too. To every patient we administrated tropicamide 1% for pupil dilatation with 20 minutes before the examination. To do ophthalmoscopy examination, all the patients have been taken in a especially dark room, used in that purpose.

The next step is the ophthalmoscopy technique, where the examiner take the lent with one hand and put her between light source and animal, at the same distances as we are talking before. Then with easy movements nearly and beyond, he, will show the tapetal reflex of the posterior pole, than very carefully , with ought losing tapetal reflex , he will move the lent until will obtain a generally view of eye fundus (retina, optic disc, choroids). The imagine obtained can be generally or can fallow in particularly different aspects as vascular aspect, optic disk aspect or retinal endothelium aspect.

2.RESULTS AND DISCUSSION

The obtained results are presented as images, captured with the video camera incorporated in ophthalmoscope.

Normal aspect of horses' eye fundus

As we say in the abstract, the study contains 21 horses, and 6 of them are foals with age until 1 year. The appreciation of morphological

eye fundus characteristics refers to his components so; we will talk about tapetal and non tapetal zone, optic disk and vascular type.

The tapetal zone: in horses is placed central and superior, and have a granular or striped aspect.

In young foals (age 2 days) the tapetum lucidum occupied a large portion of eye fundus surface, the passing to the non tapetal zone is progressive, on image appear as a horizontal band with granular aspect. That characteristic is meeting in adult horses too, but rarely.

The tapetum lucidum color varies from yellow to gray and even orange and depends by age and coat color so, in foals we seen a uniform color, light yellow excepting the passing zone to the non tapetal zone where the color is changed in orange(fig1.).

In adult horses the tapetal zone has a lower reflectivity, with a triangle form, occupied a smaller potrion of the total eye fundus surface and is represented by many colors so, we have yellow and green(fig1,2.)blue and yellow and even yellow –grey in horses with white coat ,case when we can see the coroidal vessels. The passing to the non tapetal zone is sudden.

Fig.1. Normal aspect of eye fundus in a foal with age of two days

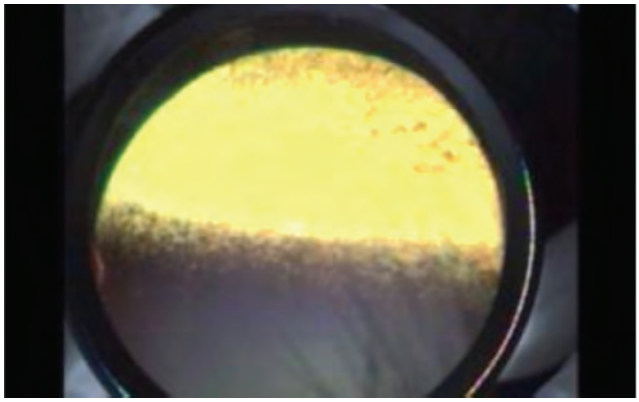

Fig.2. Normal aspect of eye fundus in a foal with age of two days

At the tapetal level, we see some darker zones, like big granulations, named Winslow stars, and represent the coroidal vessels communications with coriocapilar netting, and the granulatios are the penetration place in tapetum.The Winslow stars are visibly numerous in foals as in adult horses.

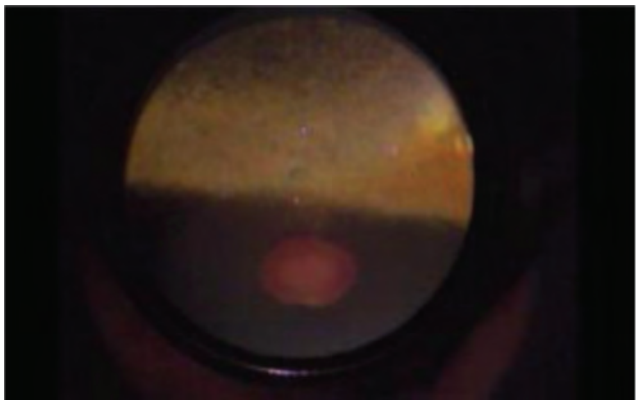

Fig.3.Normal aspect of adult horse eye fundus.

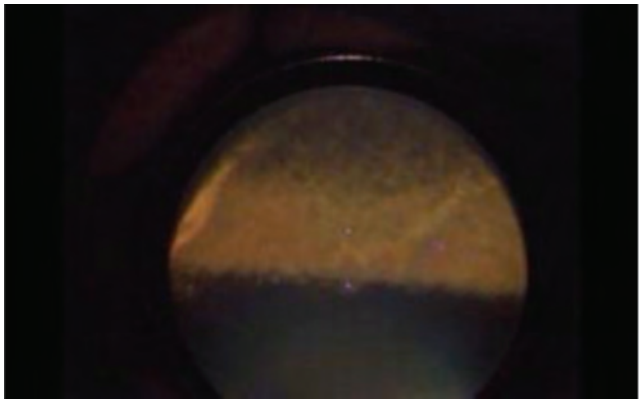

Fig.4.Normal aspect of adult horse eye fundus.

The vascular pattern include 30-60 cilioretinian vessels who starts from the optic disk in a ungle of 360 degree and pass a small area of the eye fundus. That particular aspect In horses make that ophthalmoscopic exam cant reveal the vascular design. We couldn't get a image with the vascular pattern.

The optic disk is situated in the non tapetal zone, his color is bright pink in foals and a pale pink in adults, we see in the middle the physiologic cup by a pall color(fig.3).

The shape of optic disc varies, it can be round or oval.

Normal aspect of donkey eye fundus.

Comparing with horses, donkey eye fundus is not very different, but we saw some differences.

A first difference is the color of the tapetum witch is lighter blue, uniform; very reflective .The passing to the non tapetal zone is sudden, like a compact band.

The optic disk is the same as in horses,with oval shape and pale pink(fig.5).

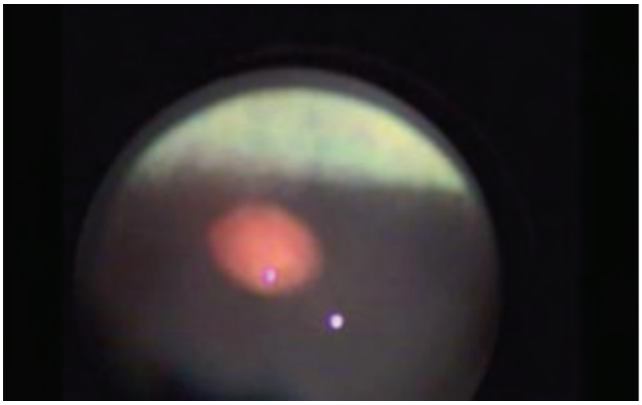

Fig .5.Normal aspect eye fundus of an adult donkey.

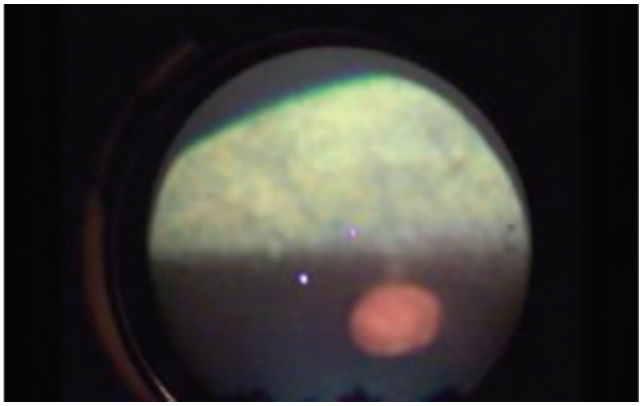

Fig .6.Normal aspect eye fundus of an adult donkey.

3.CONCLUSIONS

3.1.Horse and donkey eye fundus presents species particularity on all his components :the tapetal and non tapetal zone, optic disk and vascular pattern and we talk about color, shape and size.

3.2.The eye fundus color depends of the tapetum lucidum color and in horses is yellow with different type of green and even purple, the passing to the non tapetal zone is sluggish.

3.3.The color of donkey tapetum lucidum is blue, and the passing to the non tapetal zone is made suddenly.

3.4.The optic disk in horses is similar in donkey, the color is pink, and the shape is round or triunghiular.

3.5.In horses and donkeys, the eye fundus have a characteristic vascular pattern and on indirect ophthalmoscopy we cant see blood vessels.

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DIAGNOSTIC OF ASTEROID HYALOSIS IN DOGS TROUGH INDIRECT OPHTHALMOSCOPY TECHNIQUE

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Keywords:asteroid hyalosis,dog retina,vitreus damage

SUMMARY

Asteroid hyalosis is a degenerative status of the eye and imply on liquefaction of the vitreous body that appear as a sparkle precipitate in ophthalmoscope light.
The disease has an increased incidence in humans, dogs and chinchillas.
In the present study the ophthalmoscopic exam was made trough indirect ophthalmoscopy technique on dogs by different breed, brought to Surgery Clinic of Veterinary Medicine Cluj. The study period was 2008-2009, the animals presented different diseases not necessary from ophthalmology field. We diagnosed a few cases of asteroid hyalosis over the study, the incidence is lower if only 4 dogs from 60 presented the disease .The cause of asteroid hyalosis is incertain. Introductions From all domestic animlals the dog is the most predisposed to asteroid hyalosis.
The method used in the present study is indirect ophthalmoscopy applied with indirect ophthalmoscope Heine Omega 2C.

From all domestic animals, the dog is the most predisposed to asteroid hyaloses. The causes of asteroid hyaloids are uncertain, the specialty studies sustain a correlation with systemic disease like: diabetes, hyperlipidemia , hypertension and the same studies show that the appearance of asteroid body's is correlates with a food based on galactoses.

In the time installation, asteroid hyalosis , make damages of the vitreous body trough his liquefaction and results many white sidef spots very sparkle ,alike stars, and from this came his name,,asteroid". Asteroid hyalosis don't change only the vitreous body , sometimes had repercussion on retina, so ,the modification became an inflammation known in the special literature as ,,asteroid retinitis ".

Rarely, the disease can be observed with free eye, in most of cases the sparkle spots are diagnosed by ophthalmoscopy (direct or indirect).

About the symptoms, the animals don't accuse this body status, the visual acuity doesn't change and the diagnostic is discovered accidentally.

The characteristic symptom is detailed in humans when in case of asteroid hyalose, he complains about the appearance in visual field off a lot of stars when suddenly move his head.

In humans asteroid hyalosis have a fervency of 0, 5% and is usually unilateral with a ratio mean-female of 2:1.

The treatment of human patients is vitreotomy, but the disease doesn't disappear. In animals doesn't exist tratament.

Clinically the affected dogs, does not present visual modifications capable to affect general body status or animal behavior, the owners didn't mentioned something about those aspects.

After the indirect ophthalmic exam, from 60 dogs totally examined, only 4 was diagnosed with asteroid hyalosis .The results are presented like images captured with indirect ophthalmoscope and review defining and characteristic elements for this disease.

From 4 subjects diagnosed with asteroid hyalosis just one presents that disease bilaterally, is a rare case because in humans but in animals too is affected just one ocular globe.

Asteroid hyalosis is characterized trough small microscopic particle dispersed in vitreous body, with a star shape (asteroid) and very sparkle when is projected the ophthalmoscope light (fig.1, 2, 3).

1.MATERIAL AND METHOD

As we mention in abstract, the study was made at the Surgery Clinic on a period of two years (2008-2009), on a number of 60 dogs, adults, by different breed, sex and age, and from all this only at 4 we find specific modifications of asteroid hyahosis .Their presence to the clinic was from different reasons (plagues, castration male and female) but the main purpose was surgical intervention and beside that we made an ophthalmoscopic exam like a part of clinic exam.

The ophthalmologic diagnosis is based on indirect ophthalmoscopy and the technique is related down page.

The principle of this method is the examination of animal ocular globe, an examination made with indirect ophthalmoscope (with light source and video camera incorporated) and with a lent between examiner (ophthalmoscope) and patient. The lent is not incorporated in ophthalmoscope so, in the time of examination she must be hold with a hand by the examiner. The lent dioptrically power is 20 D and we can obtain 4-5x magnification field of view. This lent must be settle at 4-5 cm from the patient eye and at 0,5 -.0,75 m from the examiner (this in an advantage for the examiner because he keep distances from the animal).

The obtained imagine by indirect ophthalmoscopy is real and upside down. In the present study, all patients have been exanimate with ought

tranquilization, the contention was mad in a good and comfortable position for the animal and examiner too. To every patient we administrated tropicamide 1% for pupil dilatation with 20 minutes before the examination. To do ophthalmoscopy examination, all the patients have been taken in a especially dark room, used in that purpose.

The next step is the ophthalmoscopy technique, where the examiner take the lent with one hand and put her between light source and animal, at the same distances as we are talking before. Then with easy movements nearly and beyond, he, will show the tapetal reflex of the posterior pole, than very carefully , with ought losing tapetal reflex , he will move the lent until will obtain a generally view of eye fundus (retina, optic disc, choroids). The obtained image can be generally or can fallow in particularly different aspects as vascular aspect, optic disk aspect or retinal endothelium aspect.

2.RESULTS AND DISCUSSIONS

Clinically the affected dogs, does not present visual modifications capable to affect general body status or animal behavior, the owners didn't mentioned something about those aspects.

After the indirect ophthalmic exam, from 60 dogs totally examined, only 4 was diagnosed with asteroid hyalosis .The results are presented like images captured with indirect ophthalmoscope and review defining and characteristic elements for this disease.

From 4 subjects diagnosed with asteroid hyalosis just one presents that disease bilaterally , is a rare case because in humans but in animals too is affected just one ocular globe.

Asteroid hyalosis is characterized trough small microscopic particle dispersed in vitreous body, with a star shape(asteroid) and very sparkle when is projected the ophthalmoscope light (fig.1,2,3).

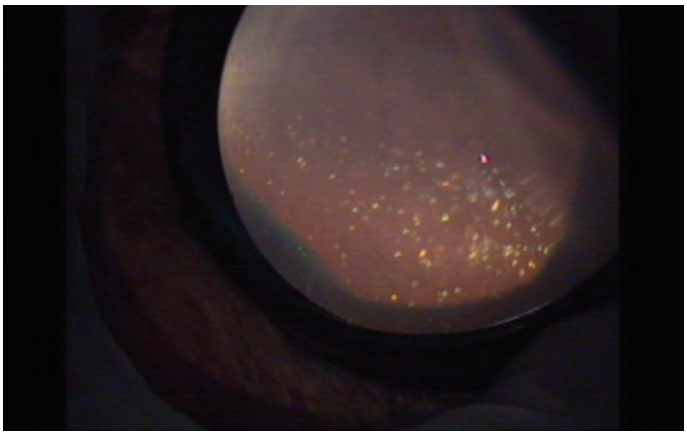

Fig.1.Asteroid hyalosis

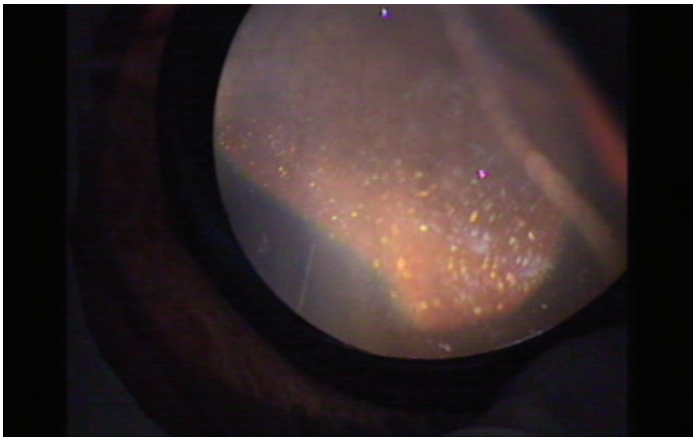

Fig.2. Asteroid hyalosis

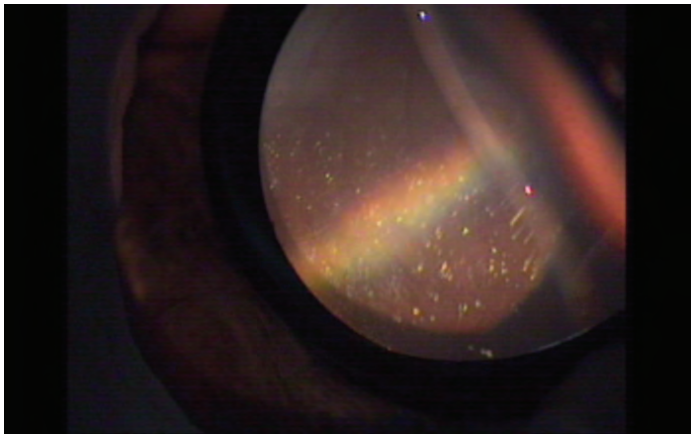

Fig.3.Asteroid hyalosis

Because of theirs sparkle properties at the ophthalmoscopic examination, rather in the moment of the projection of light beam in ocular globe, the particles appear by different colors from white,yellow to orange and green. The particles distribution is not uniform in visual field,but usually are dispersed.

A great importance is grant to those particles because can't be confused with floats and coags who can develop or located in vitreous body in a variety of diseases.

In our imagines the spots appear unequal, some are bigger on other are smaller, occupying the whole microscopic field but with a bigger density in the inferior zone.

The eye fundus elements can be seen very hard or none in asteroid hyalosis, known the fact that anatomically speaking the vitreous is situated anterior to retina and optic disk.

That 4 subjects taken in the study doesn't present macroscopic modifications of the ocular globe and visual field, the asteroid hyalosis don't imply a decrease of the animal visual field but imply a nasty discomfort if we make an extrapolate to human simptomatology.

3.CONCLUSIONS

3.1. By indirect ophthalmoscopy exam we can diagnosed easily asteroid hyalosis.

3.2. The asteroid hyalosis is a rear disease meet in animals, fact confirmed in our study because from 60 dogs examinated only at 4 we find aspects and characteristics images of that disease.

3.3. The dispersion and the number of particle is individual variable with uniform aspect with ought at some animals we saw a higher particle density in the inferior half of ocular globe.

3.4. Trough this study we manage to specify the degree of particle dispersion and visual status alteration.

3.5. Most of the times, in case of asteroid hyalosis the eye fundus cant be seen because of the loss of vitreous transparence

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THE IMPLEMENTATION OF MOLECULAR BIOLOGY TECHNIQUES IN DIAGNOSING FELINE RHINOTRACHEITIS

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Key words: Feline Rhinotracheitis Virus, implementation, PCR

SUMMARY

The identification of the Feline Rhinotracheitis Virus proved to be difficult to diagnose by traditional means because of nearly undistinguishable clinical symptoms when compared to other major feline respiratory diseases, like Feline Calicivirus and *Chlamydia psittaci*. Because of the time-consuming and/or expensive nature of most previous methods for virus identification, inaccurate and mostly clinical diagnoses were often performed, resulting in inappropriate and sometimes abusive approaches. Traditional antigen based techniques includes isolation of FHV-1 from nasal exsudates, conjunctival or oropharyngeal swabs, followed by inoculation on cell cultures, and fluorescent antibody on smear preparations from target tissues. The commonly used serological test requires biological samples to be taken 1 or 2 weeks apart in the acute and convalescent phase. The disadvantages of serological testing include the difficulty of taking sufficient quantities of blood from affected kittens, the length of time required to reach a detectable titre and the low antibody titre in convalescent cats and latent carriers. Molecular detection by PCR avoids many of the disadvantages of these other methods; PCR detection of FHV-1 is rapid, highly sensitive and very specific.

Feline herpes virus (FHV-1, feline viral rhino-tracheitis) is caused by a virus from *Herpesviridae* family, genus *Vericellovirus* (subfamily *Alphaherpesvirinae*) and can infect members of the family *Felidae* of any breed or age. FHV-1 is an enveloped virus with an icosahedral capsid that is 150 to 200 nm in diameter and contains a linear double-stranded DNA genome. The virus was first identified in 1957 and is one of two viruses currently involved in acute respiratory disease of cats; statistically, half of kittens with acute respiratory disease have FHV-1. Most cats (~70%) develop FHV-1 infections at an early age. However, kittens, immunosuppressed cats (including animals infected with feline T-cell leukaemia virus or feline immunodeficiency virus) subject exposed to various degree of stress or inadequately fed are more susceptible. Due to poor immune response, secondary infections occur massively, thus becoming a problem in animal care facilities and catteries that give refuge to many cats densely sheltered.

Infected cats develop respiratory symptoms - sneezing, nasal discharge, rhinitis (inflammation of the nose), and conjunctivitis

(inflammation of the membrane lining the eyelid). The virus also affects the reproductive tract and can cause complications during pregnancy.

FHV-1 can be spread by the discharge from an infected cat's eyes, nose and mouth, and direct contact with these secretions can be a common mode of transmission.

Several days of close contact may be enough to spread the virus.

The actual transmission can occur by contact with the animal itself or with contaminated objects that an infected cat has touched or sneezed on, such as cages, food and water bowls, litter trays and the pet owner's clothing and hands, as in most cases of infections produced by herpes-viruses.

The main problem with this infection is that many infected cats never completely sterilize and become latent carriers. Although they may not show clinical symptoms, their neurons harbour the virus and can intermittently spread the infection, being a major reservoir for new infections.

1. MATERIALS AND METHODS

Biological samples

In order to replicate the extraction matrix as well as possible, several conjunctival swabs were collected from cats showing no clinical signs. The swabs were suspended in approximately 500µl of 1% PBS (phosphate buffer saline) solution. Each of these suspensions were combined with the vaccinal strain [Tricat, *Intervet International*, containing live attenuated feline herpes virus type 1, strain G2620 A, 5.2-7.0 $\log_{10}$ PFU *(Plaque Forming Units)*] as follows: the first with 200µl of the reconstituted vaccine, and the rest with serial ten fold dilution of the viral strain.

DNA Extraction

The nucleic acids were extracted by using the DNEasy Mini Kit kit (Qiagen), according to the manufacturer’s recommendations. The DNA was recovered in a final volume of 100µl elution buffer.

Amplification

For PCR amplification, a commercial kit was used - FVR Vet (Sacace Biotehnologies), which amplify a 173bp (base pairs) from FRV 1 genome. The kit contains all the reagents required for the

amplification in a "*ready to use*" format, with a "*hot start*" polymerase to enhance stability and to prevent the occurrence of non-specific products.

To test the sensitivity to reaction, 10µl of each previously extracted DNA was amplified. Apart from those samples, positive and negative extraction and amplification controls were used to validate each of the runs.

Thermal profile consisted of initial sample denaturation/enzyme activation of 12 minutes at 95^{o}C, followed by 42 PCR cycles of 10 sec at 95^{o}C (denaturation), 10 sec at 65^{o}C (annealing), 20 sec at 72^{o}C (extension), 10 min at 72^{o}C final extension and hold at 20^{o}C until further processing.

Agarose gel electroforesis

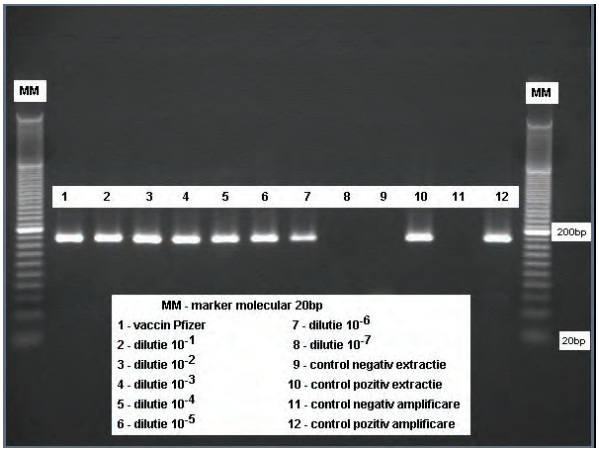
PCR products (10 µl) were loaded into the 2% agarose gel stained with ethidium bromide. Electrophoresis was performed using 1X TBE (Tris Borate EDTA) buffer and appropriate molecular weight markers (20-bp DNA ladder, BioRad Laboratoires) were used for amplicons sizes determination (Figure 1).

2. RESULTS AND DISCUSSIONS

The sensitivity of the test proved to be adequate for this kind of techniques of virus identification, being able to detect up to 10^{-7} dilution factor from the original material. Moreover, all reactions have been successfully validated, with specific bands for positive controls (extraction and amplification) and no amplification from the negative ones; this showed good performance for the extraction method and kit on the biological samples (adequate for this type of matrix), combined with low cross-contamination incidence (mainly due to the „*ready to use*" format, witch limits the preparation steps and therefore the risk of contamination).

Many studies reveal the high ranking excellence of PCR, when compared to the most commonly used techniques, in the detection of FHV in naturally acquired infection. PCR was almost twice as likely to detect FHV as VI, formerly the most sensitive test available. (Burgesser *et al.* 1999)

Fig. 1
Agarose gel electrophoresis of PCR products
MM = molecular marker 20 bp (base pairs); Line 1 to 8 = serial dilution, from 10^{-1} to 10^{-7}; Line 9 = negative control of extraction; Line 10 = positive control of extraction; Line 11 = negative control of amplification; Line 12 = positive control of amplification.



3. CONCLUSIONS

3.1. The improved rate of detection would indicate that PCR is the test of choice for the detection of FHV. Also this increased sensitivity makes PCR ideal for epidemiological studies, where population prevalence data could be determined. A positive PCR result may characterise hardly noticeable shedding or reactivation of latent virus secondary to a different primary cause.

3.2. Our study shows that a modified-live virus vaccine strain FHV will result in a positive PCR reaction and if so, how long after vaccination we can actually detect positive results? When other causes of conjunctivitis or keratitis have been eliminated, especially in chronic or recurrent disease, a positive PCR result may be more meaningful. Because the latent virus cannot be completely eliminated, a positive PCR test for FHV may give the clinician some insight into the possibility of recurring clinical signs at some time in the future if conditions are right to induce viral activation from sites of latency.

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SEROLOGICAL RESEARCHES IN AN OUTBREAK OF INFECTIOUS SYNOVITIS

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Keywords: *M. synoviae*, broiler chicken.

SUMMARY

Research has been conducted in an outbreak of infectious synovitis that has evolved in one broilers series of a farm in Timiş county. Serological examination was performed to confirm the suspicion of avian infectious synovitis. Serological examination was performed to confirm avian infectious synovitis, epidemiological and anatomoclinical suspected, in the series of broiler chickens from the studied farm. Analyzing these results, we see that at the age of 23 days (BC 1) were positive 22, 91% of the analyzed sera, and at the age of 37 days (BC 2) 26,66% have been positive. At that age, the antibody titres expressed in O.D. were much higher as the proportion of positive sera. GM of titres at 37 days old was 10,92 times higher than the GM of titres at 23 days old.

In recent years, *M. synoviae* infections are reported in chickens and turkeys, farmed in intensive system, as causing economic losses through mortality, reducing increase in weight and expenses with prevention and control. It is possible to evolve either as subclinical infections of the respiratory system or as systemic infections characterized by inflammation of the synovial membranes of joints and tendons (Cătană, 2001; Kleven, 2003).

These infections were prevalent in intensive aviculture, mainly through trade with poultry material, the vertical transmission having an important role (Cătană, 2001; Kleven, 2003).

MATERIALS AND METHODS

Research has been conducted in an outbreak of infectious synovitis that has evolved in one broilers series of a farm in Timiş county. Serological examination was performed to confirm the suspicion of avian infectious synovitis. To this end, blood samples were randomly collected from broiler chickens as follows:

- BC 1 (blood collection 1) - at the age of 23 days (48 blood samples);

- BC 2 (blood collection 2) - the age of 37 days (30 blood samples).

After the expression of sera, there were decanted into Ependorf tubes, numbered and stored in freezer until time of serological examination.

Specific antibodies were detected by ELISA test (Enzyme Linked Immunosorbent Assay), using the diagnosis kit Mycoplasma Synoviae Antibody Test Kit from Affinitech Ltd. (6).

RESULTS AND DISCUSSION

Investigations carried out in one poultry farm and the results of laboratory tests have provided important data with practical utility, respecting the evolution of avian infectious synovitis, in broiler chickens from that outbreak.

Serological examination was performed to confirm avian infectious synovitis, epidemiological and anatomoclinical suspected, in the series of broiler chickens from the studied farm.

The results of this exam, performed by ELISA test, are shown in Table 2. After the interpretation of reactions and the processing of results, according to the interpretation soft of the FlockChek® Avian MS Antibody Test Kit, for each collection there were assigned: the titre group, the minimum titre, the maximal titre and the geometrical mean (G.M.). The titres were expressed in optical densities (O.D.).

In the first blood sample collection (BC 1), at 23 days old, there were identified 5 titres groups (0-4), minimum titre was of 0 O.D. and maximal titre was of 3000 O.D.

In the second blood sample collection (BC 2), at 37 days, there were identified 4 titres groups (0-3), minimum titre was of 15 O.D. and maximal titre was of 2293 O.D.

Analyzing these results, we see that at the age of 23 days (BC 1) were positive 22, 91% of the analyzed sera, and at the age of 37 days (BC 2) 26,66% have been positive. At that age, the antibody titres expressed in O.D. were much higher as the proportion of positive sera. GM of titres at 37 days old was 10,92 times higher than the GM of titres at 23 days old.

The seroconversion evolution shows consecutive postinfectious immune response to the aggressive character of mycoplasmas and their active implication in the pathological process.

Table 1

Rezultatele examenului serologic efectuat prin testul ELISA
The results of serological exam performed by ELISA

No. Crt.	BC 1/23 days		BC 2/37 days	
	Titre group	Samples number	Titre group	Samples number
1	0	37	0	22
2	1	4	1	5
3	2	4	2	1
4	3	2	3	2
5	4	1	4	0
7	**Maximal titre**	**3000 O. D.**	**Maximal titre**	**2293 O. D.**
8	**Minimum titre**	**0 O. D.**	**Minimum titre**	**15 O. D.**
9	**Titres geometrical mean**	**13**	**Titres geometrical mean**	**142**

The results of serological examination confirmed the presence of infection with *M. synoviae*, whose suspicion has been established by the epidemiological and anatomoclinical exams.

Meanwhile, the results provided by this exam demonstrate a postinfectious specific immune response.

Values and the proportion of positive titres obtained at the age of 23 and 37 days reveal both the involvement of *M. synoviae* in the etiology of the disease and its infectiousness, by horizontal transmission.

The values of the antibodies anti *M. synoviae* titres, expressed in O.D., are similar to values reported by other authors (Nascimento et al., 2005; Takase et al., 2000).

Recorded high levels of antibodies anti *M. synoviae* in broilers, at the age when the disease outbreak evolved, confirm the existence of postinfectious immune response.

The value of geometric mean titres is of 10,92 times higher at 37 days old than at 23 days old, and higher proportion of the obtained positive titres reveals both M. synoviae involvement in disease etiology and its infectiousness, by horizontal transmission.

The results of this research are similar to those published by other authors, such recommends the serological monitoring of broiler flocks as an efficient control of infection and allows the start in time of preventive treatment when it is necessary (Bencina et al., 1988; Takase et al., 2000).

Existence of *M. synoviae* infection in flocks of broilers is a serious concern for practitioners in the field of aviculture because of significant economic losses that may occur in this entity.

CONCLUSIONS

Serological examination has marked out specific antibodies and seroconversion phenomenon characteristic of an evolutionary infectious process.

1.1. Titres of antibodies expressed in O.D. and the geometrical mean at BC 2 (37 days) were much higher than in BC 1 (23 days) as a consequence of local infections, but, especially, of the systemic infection.

1.2. Imunoenzimatic test can be used in the diagnosis of infections with *M. synoviae* in broilers, when there is an epidemiological and anatomoclinical suspicion.

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EPIDEMIOLOGICAL AND ANATOMOCLINICAL RESEARCHES INTO AN OUTBREAK OF MYCOPLASMAL SYNOVITIS

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Key words: *M. synoviae*, synovitis**.**

SUMMARY

In one poultry farm of Timiş county, in a series of imported broilers, there were reported characteristic symptoms of mycoplasmal synovitis, losses by mortality and by reducing increase in weight. Epidemiological examination was carried out as epidemiological investigation, having the following objectives: identify the source of infection, detecting of the favourable factors and the ways of disease spreading, mortality and increase in weight. Clinical examination was done daily after the age of 14 days old, age at which began to appear first symptoms of the disease. Anatomopathological examinations were performed twice per week in broilers corpses, being noted the anatomopathological gross lesions.
Epidemiological examination carried out has marked out the possibility of horizontal and vertical transmission between broilers of the same flock and further by selling broilers to people, the infection could be transmitted to other birds in their household. Weekly cumulative mortality of broilers in the two shelters had maximum values in the IV^{-th} and V^{-th} weeks. At the end of the growth period, cumulative mortality was of 24,49% for A shelter and of 25,55% for B shelter. Symptoms occurred in broilers after 14 days of age. Chickens presented depression, poor appetite, adynamia, lameness, limps, uni- or bilateral arthritis in tarso-metatarsian joints. These joints have been increased in volume, fluctuating and sensitive. In the anatomopathological examination of broilers cadavers**,** performed twice per week, there were observed the following gross lesions: exudative synovitis in the joints of fangs and fans. In incipient stage of arthritis, there is a viscous creamy to gray exudate, and in chronic evolution there is a caseous exudate that involves tendon synovial sheats.

In birds, mycoplasmal infections are widespread in all countries that practice intensive aviculture, depending on the intervention of favourable factors. They cause significant economic losses, whatever the clinical form evolving, by reducing the production of eggs, meat and by mortality (Bencina et al., 1988; Cătană, 2001).

Intense trade of poultry material contributed to the spread of avian infectious synovitis from the Northern American continent to other continents, the disease being known in all countries that practice intensive aviculture. The rapid spread of that disease has been favored by horizontal transmission, but especially by way of vertical transmission, and from the breeding flocks too (Kleven,1983; Nascimento et al., 2005).

MATERIALS AND METHODS

In one poultry farm of Timiş county, in a series of imported broilers, there were reported characteristic symptoms of mycoplasmal synovitis, losses by mortality and by reducing increase in weight.

Epidemiological examination was carried out as epidemiological investigation, having the following objectives: identify the source of infection, detecting of the favourable factors and the ways of disease spreading, mortality and increase in weight.

Losses through mortality were measured and interpreted by cumulative mortality. This indicator was used to express the losses, because it allows the dynamic evolution pointing of the mortality, as relative value obtained by reporting the absolute value of this indicator to the number of broilers from the series, in the first day when the farm was populated, being at risk for the entire period of growth.

Clinical examination was done daily after the age of 14 days old, age at which began to appear first symptoms of the disease.

Anatomopathological examinations were performed twice per week in broilers corpses, being noted the anatomopathological gross lesions.

RESULTS AND DISCUSSION

In that farm, at time when it was populated with that broilers series, there were three halls of adult layers, farmed intensively in battery cages, and two halls of broilers, aged 22 days old.

In that farm the biosecurity measures and general prevention are partially applied.

Epidemiological examination carried out has marked out the possibility of horizontal and vertical transmission between broilers of the same flock and further by selling broilers to people, the infection could be transmitted to other birds in their household.

Broilers from that series where the disease occurred and progressed, in number of 12 600, were raised on soil, in two shelters of 6 300 chickens in each one. Given granulated forage have corresponded from qualitative and quantitative point of view.

Hygiene conditions were poor, the main parameters (humidity, air currents, gas) were above admissible limits, the values being higher after the age of 3 weeks old.

In Table 1 are presented the losses expressed by cumulative mortality. Weekly cumulative mortality of broilers in the two shelters had maximum values in the IV^{-th} and V^{-th} weeks. At the end of the

growth period, cumulative mortality was of 24,49% for A shelter and of 25,55% for B shelter. These values correspond to data from the scientific literature, most authors considering that cumulative mortality is exceeding 20% in mycoplasmal synovitis in broilers (Levisohn and Kleven, 2000).

Cumulative mortality points losses through mortality as relative values, as percentage or decimal value, the last method being used in this study.

Table 1

Mortalitatea cumulativă săptămânală la puii de carne
Weekly cumulative mortality of broilers

Week	Shelter A		Shelter B	
	No. Death	**%**	**Nr. death**	%
I	203	**3,22**	211	**3,34**
II	235	**3,73**	243	**3,85**
III	241	**3,82**	257	**4,08**
IV	302	**4,79**	311	**4,94**
V	315	**5,00**	322	**5,11**
VI	247	**3,92**	266	**4,22**
Total	1543	24,49	1610	25,55

Symptoms occurred in broilers after 14 days of age. Chickens presented depression, poor appetite, adynamia, lameness, limps, uni- or bilateral arthritis in tarso-metatarsian joints. These joints have been increased in volume, fluctuating and sensitive. For these reasons, broilers did not move to drink water and to feed, and that bring to progressive debility. Some chickens, besides the locomotion symptoms, had dyspneea and tracheal rales.

KLEVEN and FLETCHER observed the clinical signs, in natural infection of broilers, beginning at 1 week old, even if the acute infection usually appears at 4-16 weeks old in broilers (Kleven and Fletcher, 1983).

In some chickens, after 4 weeks old, the extension of the tendon sheaths was observed and it was followed by the impossibility of walking and the extension of the affected limb. This modification was usually unilateral.

In infectious avian synovitis, in the affected chickens were observed general signs, localized symptoms to the joints, and respiratory symptoms indicating the location of infection with *M. synoviae* and in

some segments of the respiratory system, issues reported by other authors (Lockaby et al., 1998).

In the anatomopathological examination of broilers cadavers**,** performed twice per week, there were observed the following gross lesions: exudative synovitis in the joints of fangs and fans. In incipient stage of arthritis, there is a viscous creamy to gray exudate, and in chronic evolution there is a caseous exudate that involves tendon synovial sheats. Frequently, it was observed the rupture of the gastrocnemius tendon and the destruction of joints surfaces. Besides the lesions of locomotor system there were observed fibrocaseous aerosaculitis, hepatomegaly, splenomegaly and sternal bursitis.

In the scientific literature it is mentioned that infection is characterized by an exudative synovitis which is represented, in the first stages, by the inflammation of membranes of the synovial sheaths from the region of knees, phalanges, stern and mandible, expressed by an accumulation of a viscous creamy to gray exudate which becomes caseous in chronic infections (Paul, 1996).

KERR and OLSON revealed, in experimental infections, the erosive character of the arthritis caused by *M. synoviae*: at 165 days postinfection, the joints surfaces being completely destroyed and replaced by fibrouses processes with adhesive tenosynovitis, followed by the anchylosis of joints (Kerr and Olson, 1967).

CONCLUSIONS

1.1. In the studied outbreak it was reported a disease with characteristic symptoms of the infectious mycoplasmosis, that has evolved in the form of avian infectious synovitis.

1.2. In this outbreak the epidemiological investigation confirmed the vertical transmission of disease through hatching eggs and subsequently the horizontal transmission in the flock of broilers, after populating the two halls.

1.3. Cumulative mortality has evolved in the characteristic limits of this disease, as an important epidemiological indicator of the epidemiological investigation.

1.4. Clinical examination revealed the main symptoms of this disease, located in limbs, and the respiratory symptoms.

1.5. Anatomopathological examination marked out the characteristic lesions at tibio-tarso-metatarsal joints and tendons of gastrocnemius muscles.

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EFFECT OF LOW-LEVEL LASER THERAPY ON WOUND HEALING IN DOGS

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Key words: LLLT, wounds, dog.

SUMMARY

The authors studied the effect of laser radiations with wave length of 635 nm on wound healing in dogs. The treatment was applied according to a protocol for 9 days at a power of 15 mW, for 300 seconds and from a distance of 0.5 cm from the wound, using a multiple probe with 5 diodes placed in the shape of a star. The treatment was done daily starting with the first post surgery day.

The treatment was done to 10 dogs (bitches) with post operation wounds. During the treatment we monitored the clinical evolution of the wounds by taking photographs of them and by digital processing of the images; at the end of the observation period biopsy samples were collected randomly and processed histologically.

The results obtained after 9 days of treatment showed a favourable clinical evolution of healing. On day 10 post surgery, 90-100% of the animals treated with laser showed fully healed wounds in the absence of any local or general treatment with disinfectants, chemotherapy or antibiotics. The biopsy samples showed the presence of the granulation conjunctive tissue and the full epithelisation of the wound 10 days after surgery.

In conclusion, we consider that laser therapy at 635 nm, applied according to the protocol established experimentally, stimulates the process of wound healing and shortens the period of hospitalization.

Soft tissues therapy with low-level laser beams (wavelength between 630-920 nm) has been used by the human medicine as early as from the 70s, being considered to be an atoxic, non-invasive and non-polluting therapy. The 1967 paper of Mester is the first mentioning the beneficial effects on the tissues of photostimulation with laser beams. The photostimulation with laser of the soft tissues determines a local and profound (4-5 cm) anti-inflammatory, regenerative and analgesic action. The mechanism of action and the modalities of tissue stimulation have been presented by the author in a previous paper (Coman et al., 2004).

Subsequent research have determined experimentally the stimulating effect of he low-level laser beams of the fibroblasts, macrophages and keratinocytes in cell cultures, as shown by the increase of the amount of synthesized collagen, by the stimulation of cell replication, by the stimulation of calcium ion build-up in the

mitochondria, by the stimulation of mitochondria replication in the epithelial and conjunctive cells, by myofibroblasts replication, by the stimulation of the angiogenesis factor synthesis, by the stimulation of endorphins synthesis, etc. (Alena et al., 2003; Bolton et al., 1991; Lubmart et al., 1993; Rigau J. et al., 1996; Young et al., 1991).

In the clinical practice, not all treated cases produced the expected results. Some papers showed the failure of using laser radiations to treat soft and hard tissues (aponeuroses, tendons, muscles, bone exostoses). Lucas C. et al. (2002), in a review of LLLT effect in wound treatment in animals and in human clinics, showed that 37% of the published papers didn't report positive results of the treatment.

The paper of Hallman H.O. et al. (1998) showed that energy intensities higher than 9.6 J/cm^2 have inhibitory effect on the fibroblasts in cell cultures preventing their replication. These studies show the imperative of establishing treatment protocols for each single disease based on experiments which can provide for a maximal efficiency of the treatment.

Under experimental conditions, Coman T. et al. (2006), have determined the parameters of LLLT treatment of the wounds in pets function of the type of disease, biological substrate, skin thickness etc. The use of these parameters demonstrated the stimulating action in the process of aseptic wound healing in rabbits and he beneficial effects obtained by the shorter period of hospitalization and by the fast healing without the use of local or general treatments.

Surgical wounds are often met in the veterinary medicine. Ionescu P. (1999) shows the experience of the researchers from the Army Hospital in LLLT treatment of tissue ailments in animals.

In the case of atone wounds, with bacterial contamination, the treatment takes a longer period and requires the use of antibiotics and chemotherapy for general and local treatments (Petersen et al., 1999).

1. MATERIAL AND METHOD

The treated **animals** were dogs of common breeds, of different age and sex, brought to the clinic of the faculty. A number of 10 bitches, which were surgically treated by laparotomy due to ovariectomy, were treated post surgery with laser radiations according to the protocol for aseptic wounds treatment. The treated animals were clinically healthy and the haematological and blood biochemical parameters were within the physiological range. The animals started the treatment during the early hours after surgery. The animals treated with laser radiations

didn’t receive any local or general treatment which to favour wound healing.

The **laser** used for treatment has been produced by the National Institute of Optoelectronics. It was fitted with a pen-type probe, with intermittent emission with the wave length (λ) 830 nm and with a multiple probe with the wave length 635 nm (5 diodes placed in the shape of a star, each diode with the power of 3 mW (fig. 1).

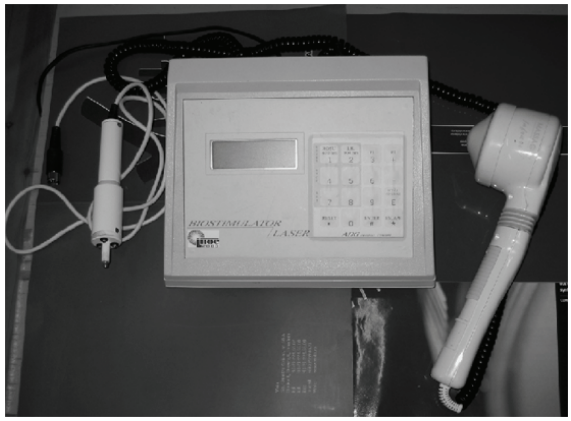

Fig. 1. Low-level laser system

The **irradiation parameters** selected for the treatment of aseptic and septic wounds were as follows:

- continuous emission
- energy density 5 J/cm^2
- time of irradiation: 300 seconds
- power density: 15 mW
- frequency: 7.000 Hz
- treatment frequency: daily
- period of treatment: 9 days
- distance of irradiation: 0,5 cm.

METHODS OF INVESTIGATION

• The clinical examination was done on a daily basis. The body temperature of the animals was monitored and the wounds have been photographed. The photos were processed digitally on computer to show the intensity of colour intensity absorption. The better was wound healing, the highest was light absorption. In the areas with haemorrhages, blood clots or unhealed places, colour intensity was

below 30.000 a.u, while in the healed areas the colour intensity ranged between 40.000-60.000 a.u.

• The histological examination was done randomly, by processing biopsy samples taken at the end of the treatment. The biopsy samples were fixed in neutral saline formalin processed histologically and stained using the usual trichromic methods.

2. RESULTS AND DISCUSSION

The clinical observations and the photos processed digitally have shown the first beneficial results of the laser treatment during the second after surgery (fig. 2). 48 hours after surgery, the wound margins were close, the wound was dry, there were no oedema, but there was a slight marginal erythema. Figure 3 shows the graphical distribution of the colour intensity absorption, which varies widely between 5.000 a.u. and 55.000 a.u., with two marginal peaks given by the suture points.

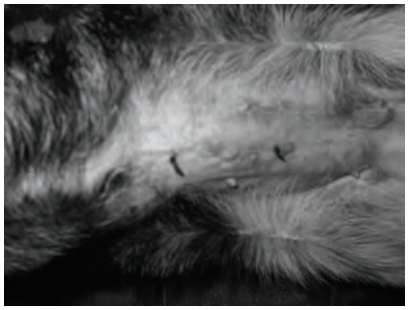

Fig. 2. Aseptic surgical wound, 48 h after surgery and after the third treatment with LLLT

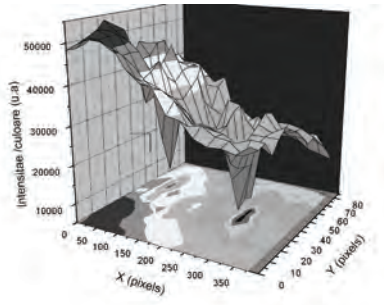

Fig. 3. Distribution of the colour intensity of an aseptic surgical wound, 48 h after surgery and after the third treatment with LLLT

After 72 hours from surgery (the fourth treatment with LLLT) the wound appears uniform, with not exudates or oedema, showing a local inflammatory reaction at the suture points (fig. 4).

The graphical analysis of the colour intensity absorption shows an almost uniform intensity of colour absorption of the wound, except for the two suture points where colour absorption is below 20.000 a.u. (fig. 5).

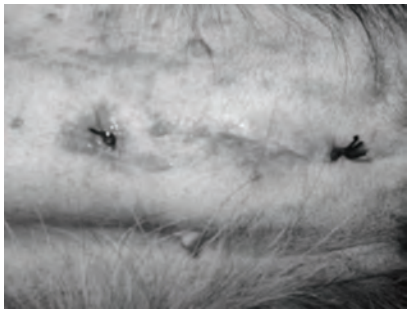

Fig. 4. Aseptic surgical wound, 72 h after surgery and after the fourth treatment with LLLT

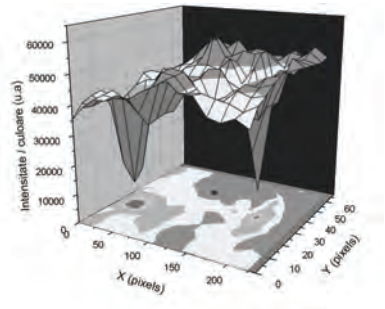

Fig. 5. Distribution of the colour intensity of an aseptic surgical wound, 72 h after surgery and after the fourth treatment with LLLT

On day 8 after surgery (9th treatment with LLLT) the wound appears epithelised except for the suture points, one of the points being almost eliminated (fig. 6). The graphical analysis of the colour intensity absorption shows a constant absorption starting from the left suture point, which is almost eliminated, with values ranging between 40,000 a.u. and 50,000 a.u., except for right the suture point where the absorption of the colour intensity was 10,000 a.u. (fig. 7).

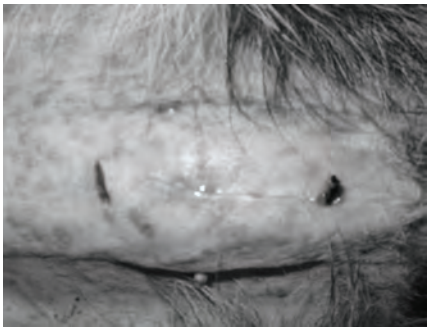

Fig. 6. Aseptic surgical wound, 8 days after surgery and after the 9th treatment with LLLT

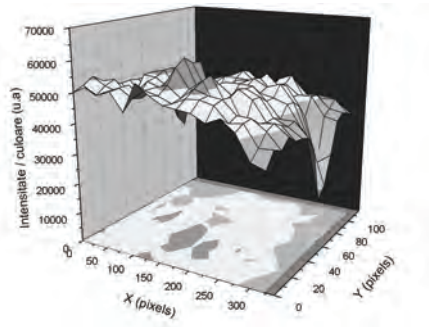

Fig. 7. Distribution of the colour intensity of an aseptic surgical wound, 8 days after surgery and after the 9th treatment with LLLT

On day 10 after surgery (9th treatment with LLLT) the wound appears fully healed; it is elastic, with no adherences or poor healing (fig. 8).

Under the conditions of LLLT treatment of the septic wounds healing was „*per primam intentionem*". The analysis of the colour intensity absorption shows an almost uniform intensity of colour

absorption with small variations between 30,000 a.u. and 45,000 a.u. This shows an almost complete epithelisation of the wound (fig. 9).

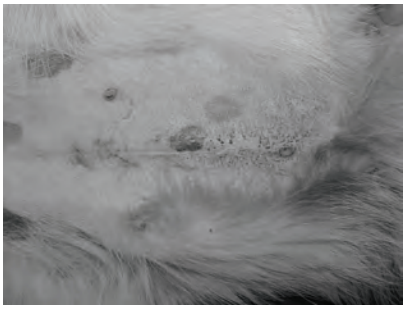

Fig. 8. Aseptic surgical wound, 10 days after surgery and after the 9th treatment with LLLT

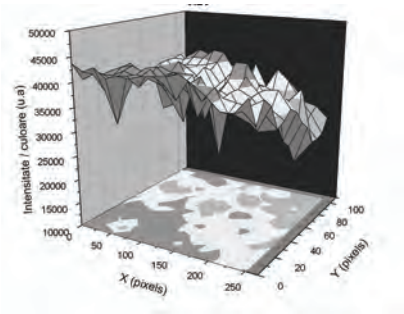

Fig. 9. Distribution of the colour intensity of an aseptic surgical wound, 10 days after surgery and after the 9th treatment with LLLT

The histological examination of the biopsy sample shows a full epithelisation with epidermis and dermis regeneration (fig. 10).

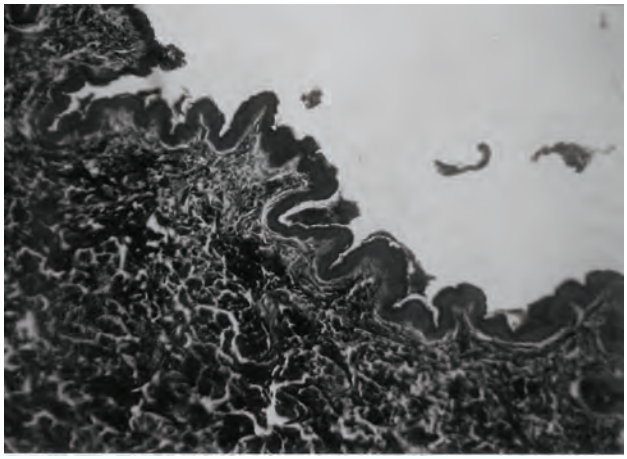

Fig. 10. Aseptic surgical wound, 10 days after surgery; Trichromic Mallory staining; 10×

The healing of the aseptic wounds can be monitored by the graphical representation of the colour intensity absorption (Fig. 11). The diagram shows the evolution of the healing 48h, 72h, on day 8 and on day 10 after surgery.

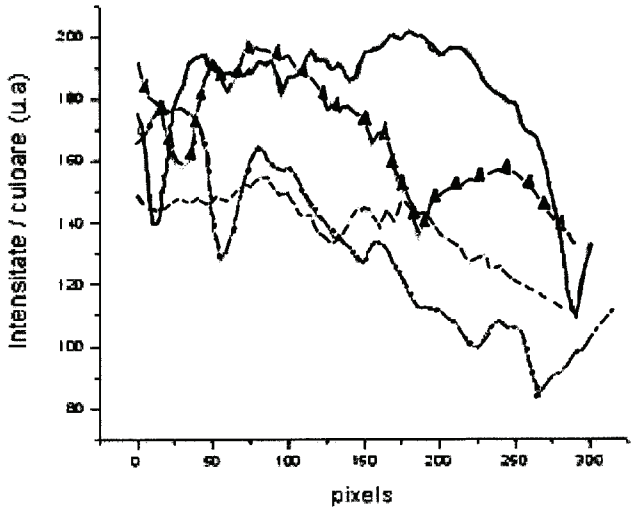

Fig. 11. Bidimensional graphical representation of the healing process „*per primam intentione*m" in aseptic surgical wounds

The continuous curve shows colour absorption 10 days after surgery, when epithelisation is almost complete except for the suture points. Wound healing was much accelerated due to the stimulating action of the laser radiations of 635 nm. The literature shows that „*per primam intentionem*" healing of the wounds takes 14-21 days. In the case of the laser treatment healing was 10 days shorter, which means a shorter period of hospitalization with a fast recovery of the animals.

3. CONCLUSIONS

3.1. The healing of aseptic wounds was shortened by 10 days post surgery by low-level laser therapy (λ=635 nm).

3.2. Irradiation must be done according to a protocol established experimentally for the type of treated ailment.

3.3. The laser treatment of aseptic wounds was done with no local or general medicine treatment, which decreases the cost of treatment but it doesn't excludes the possibility of applying the medication.

3.4. The laser treatment of the wounds ensures:

- fast healing of the wounds;
- proper draining of the post surgery oedema;
- formation of an elastic scar with no adherences;
- lower local pain.

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SOME HISTOPATHOLOGICAL ASPECTS OF THE LIVER IN BROILER CHICKENS AGED 34-36 DAYS

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1. DSVSA Prahova; 2. Faculty of Veterinary Medicine Bucharest

Key words: broiler chiken, liver, histology.

SUMMARY

The patho-anatomical study of the liver coming from broiler chickens raised in intensive systems was interested in catching the macroscopical aspects especially the microscopical image which would define the problematic of slaughter losses due to feeding in the seven days before slaughtering. Therefore, from the total of daily slaughtered chickens, approximately 24000-26000 individuals, at least 500-1000 of them presented enlarged liver with pronounced friability. Histologically, a massive infiltration with lymphoid cells was noticed, evidentiating the hepatic macrophages and appearance of necrosis islets, along hepatosteatosis territories. The uncomercial, poor quality image, determined the slaughter products to be confiscated and send to be incinerated, causing great economical losses.

A series of nutritional problems that cause the abnormal synthesis, use or mobilization of fats stand at the origin of the fatty liver syndrome. The massive accumulation of lipids in the hepatocytes causes important metabolic alterations that end with cellular death, expressed with the debute of necrotic processes [2].

The start of hepatosteatosis is macroscopically detectable in the enlarged, friable, earthy-yellowish, oily in section, liver [1,3].

Microscopically, the hepatocytes contain lipidic vacuoles showing no color when colored with usual methods. The vacuoles may have different sizes, sometimes even deforming the cell. This kind of cells can be located in the center of the lobule, at the periphery or randomly in the hepatic parenchyma [4,6].

The histostructural alterations caused by the accumulation of lipids in hepatocytes determine increased frequency of liver ruptures [5].

1. MATERIAL AND METHOD

The researches histologically studied the fatty liver in chickens aged 34-36 days. The study was done on liver samples obtained after the slaughtering of broiler chicken in a chicken slaughter farm in Prahova county.

These chickens were floor-raised on permanent litter, in production houses with 24000-26000 chickens capacity.

The birds were fed with combined fodder, respecting a scheme that is in harmony with the particularities of the age categories, that meaning “stater” fodder until they reach 15 days and then grower fodder until 34-36 days, daily supplemented with a Vibromax type product.

The liver taken was fixed in saline neuter formaline and introduced in paraffin for further processing. The blocks of paraffin were sectioned into 6 microns thick sections and colored with Hematoxylin-Eosin and Giemsa staining methods. For each of the three lobes, several permanent histological sections were done, that were later on examined under a Nikon microscope and photografied.

2. RESULTS AND DISCUSSIONS

Macroscopically, a part of the enlarged liver samples appear yellowish with hemorrhagical areas and another part seems to be just slightly enlarged and red-orangy. Both tipes of samples present an accentuated friability, which determines the liver to be held from commercialization. In some cases, the liver was ruptured. Though, the fatty aspect of the section was not heavily marked.

Histostructurally, the hepatic territories vary very much as image, and in the affected areas, they interest more the right lobe, which has the most extent.

The capsule appears slightly thickened, presenting a moderate fibrosation. Near it, agglomerated lymphoid cells can be observed, cells that create a compact marginal area.

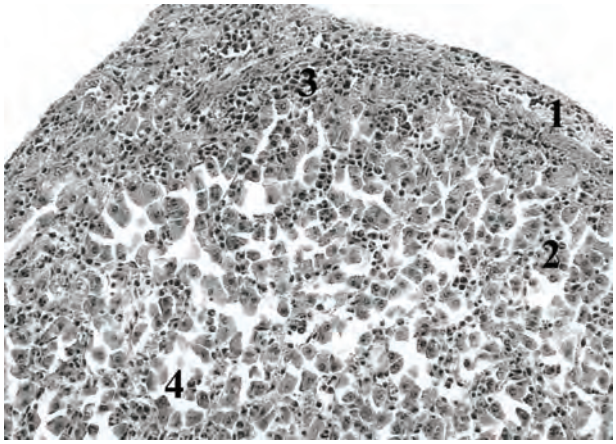


Figure 1. Presence of marginal territories with edema in the liver parenchyma/ HE, ob. 20x
1. Capsule; 2. Hepatocytes; 3. Lymphoid infiltrations; 4. Edema.

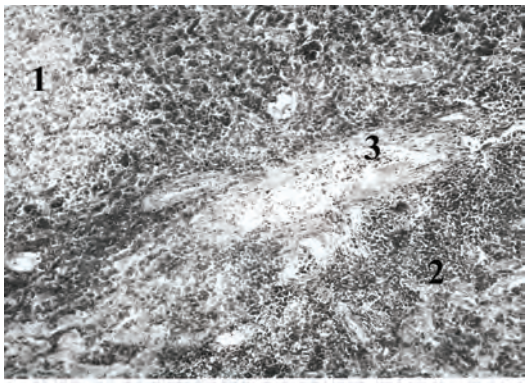
Towards the periphery of the hepatic parenchyma the groups of hepatocytes appear to be drifting away from the basal membrane of the sinusoids, indicating the presence of some marginal territories with edema. These areas of edema are limited and do not always accompany the other anatopathologic alterations (Figure 1).

The edema of the hepatic parenchyma has no macroscopic correspondent, but the frequent rupture of the liver implies its involvement in this phenomenon. As we head to the deepness of the hepatic liver, the edema reduces, yet appears blended with a moderate lymphoid infiltration, uniformly spread.

In the hepatic parenchyma, alongside normostructural areas, there are massive lymphoid infiltrations especially near the centrolobular veins and near the porto-biliar area. Here and there, among the lymphoid cells, islets of hepatocytes with vacuolized, foamy cytoplasm, are seen (Figure 2).

Figure 2. Lymphoid infiltrations near the porto-biliar area/ Giemsa, ob. 10x

1. Hepatosteatosis area;
2. Lymphoid infiltration;
3. Porto-biliar area.



Frecquently, hepatic macrophages are remarked, sometimes tending to group or present as siderocytes, after accumulating pigment, near the porto-biliary space. The neighbouring hepatocytes suffer discrete necrotic processes.

The mononuclear infiltration can occupy vaster areas, where a maximum density of lymphocytes, plasmocytes and macrophages is seen, along with the presence of neutrophyles and eosinophiles. Sometimes, all these present a tendency of limphonodular grouping. Both to the periphery of these territories as well as in the central area, numerous hepatocytes with foamy, optic clear cytoplasm can be seen. The accentuated vacuolar character indicates the location of lipids, although the intense steatotic aspect due to large, agglomerated, multiple, round vacuoles is not particulare to the studied cases (Figures 3, 4).

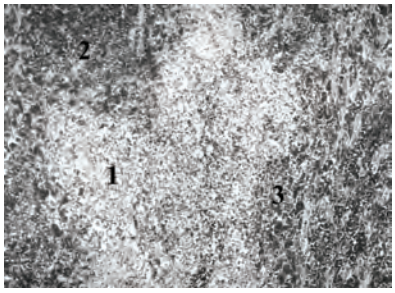


Figure 3. Hepatosteatosis territory/ Giemsa, ob. 10x

1. Hepatosteatosis area; 2. Lymphoid nodule; 3. Diffuse lymphoid infiltration.

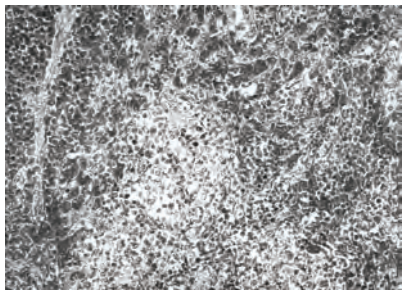


Figure 4. Detail of hepatosteatosis territory neighbouring with large area of lymphoid infiltration/ Giemsa, ob. 20x

Among the hepatocytes with foamy cytoplasm, the dilatation of the sinusoids is visible, suggesting eritrocitar agglomerations.

3. CONCLUSIONS

3.1. In the case of applying of personal nutritional schemes that have vitamine supliments, the presence of enlarged, friable, predominantly yellowish or red-orangy liver was observed in 8-10% of the slaughtered birds.

3.2. Histostructurally, in the studied samples, the presence of edema in the periphery of the hepatic parenchyma is observed, alongside a massive lymphoid infiltration near the controlobular veins and in the porto-biliar space, manifesting even a tendency of limfonodular organization.

3.3. The hepatocytes with fatty, foamy cytoplasm, are dispersed in the parenchyma and interfere with areas in which agglomerations of eritrocytes that expand the sinusoids are observed.

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TRICHOTHECENES – ANIMAL AND HUMAN HEALTH RISK

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Key words: trichothecenes, T-2 toxin, NIV, DON, toxic effects

SUMMARY

Trichothecenes are a class of more than 180 structurally related sesquiterpenoid metabolites produced by food borne and environmental fungi. More than 40 naturally occurring trichothecenes are produced by fungi belonging to the genus *Fusarium*. Trichothecenes are classified into four groups (A, B, C and D), the most important being the ones belonging to the A and B group: T-2 toxin (the most toxic, A type trichothecene), nivalenol (NIV, less toxic than T-2 toxin, B type trichotecene) and deoxynivalenol (DON, the most prevalent trichothecene, contaminating grain worldwide, B type trichothecene). Toxicological effects associated with trichothecene mycotoxin poisoning in animals and humans include anorexia, gastroenteritis, emesis, hematological disorders, cytotoxicity and immunosuppression. This paper is an overview of the chemical and biochemical characteristics, toxicity, health impact and legislative limits of the most common trichothecenes found in cereals that pose a risk to the animal and human health.

Trichothecenes are mycotoxins produced mostly by members of the *Fusarium* genus, although other genera (e.g. *Trichoderma, Trichotecium, Myrothecium and Stachybotrys*) are also known to produce these compounds (Peraica, 1999). *Fusarium* species are field fungi with a world-wide occurrence and the genus is probably the most important toxin-producing fungi of the northern and temperate regions (Ueno, 1983, quoted by Eriksen, 2003). These fungi infect cereals in the field such as wheat, rye, oat, maize and barley. They grow and sporulate both in soil and on plants. The degree of infection will depend on various factors, for example temperature, humidity, rainfall during anthesis (flowering), and at crop harvest, soil treatment and crop rotation (Eriksen, 2003).

Trichothecenes are tricyclic sesquiterpenes that contain a double bond between carbons 9 (C-9) and 10 and a 12, 13-epoxide ring, and are thus designated as 12, 13-epoxytrichothec-9-enes. They are also characterized by various patterns of oxygenation and esterification at positions C-3, C-4, C-7, C-8 and C-15 (Desjardins and Proctor, 2007).

Trichothecenes are divided into four categories according to their functional groups. Type A has a functional group other than a keto group at C-8. This is the largest group and includes toxins like T-2

toxin, HT-2 toxin and diacetoxyscipenol (DAS). Type B trichothecenes has a keto group at C-8 and includes the most widespread trichothecene DON and toxins like 3-aDON, NIV, and Fusarenon-X. The third category (Type C) has a second epoxide ring at C-7,8 or C-9,10 and toxins from the fourth group (Type D) contains a macrocyclic ring between C-4 and C-15 with two ester-linkages (Eriksen, 2003, Mărioara Drugă, 2007). These structural characteristics are shown in Fig.1 and 2 below:

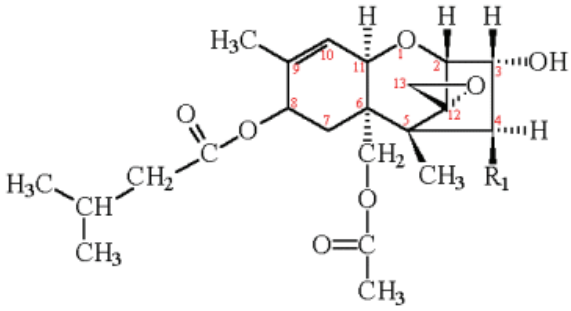

Fig. 1: Structural formula of type A trichothecenes:
T-2 (R1 = OAc); HT-2 (R1 = OH)
http://services.leatherheadfood.com/mycotoxins/item.asp?sectionid=3&mytype=expert&number=3&fsid=27

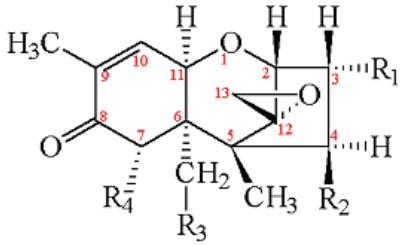

Fig. 2: Structural formula of type B trichothecenes:
DON (R1 = OH, R2 = H, R3 = OH, R4 = OH)
NIV (R1 = OH, R2 = OH, R3 = OH, R4 = OH)
3-AcDON (R1 = OAc, R2 = H, R3 = OH, R4 = OH)
15-AcDON (R1 = OH, R2 = H, R3 = OAc, R4 = OH)
FUS-X (R1 = OH, R2 = OAc, R3 = OH, R4 = OH)
http://services.leatherheadfood.com/mycotoxins/item.asp?sectionid=3&mytype=expert&number=3&fsid=27

From more than 180 trichothecene compounds identified, only a few are toxic to animals and humans. The most important ones that pose a risk to animal and human health are T-2 toxin, nivalenol and deoxynivalenol. These three metabolites are reviewed in this paper.

1. NATURAL OCCURRENCE

Deoxynivalenol (DON), also known as "vomitoxin", is a mycotoxin classified as a type B trichothecene which is produced mainly by *Fusarium graminearum* and *Fusarium culmorum*. DON occurs in toxicologically relevant concentrations in cereals and grains worldwide (Pestka, 2007).

In 2001, Joint FAO/WHO Expert Committe on Food Aditives and Contaminants (JECFA) prepared a compilation of the data from surveys on DON in random samples of cereals and in an world-wide average. DON has been found in 57% of the wheat samples, 40% of the maize samples, 68% of the oats samples, 59% of barley samples, 49% of rye samples and 27% of the rice samples analysed. DON was also found in wheat and maize products, for example flour, bread and breakfast cereals. The concentrations of DON in random cereal samples showed a large annual variation, concentrations ranging from below the detection limits (5-50 μg/kg) to more than 30 mg/kg (JECFA, 2001).

The two *Fusarium* species are plant pathogens and cause outbreaks of *Fusarium* head blight (also called wheat scab). The most serious outbreaks of the disease occur in years with heavy rainfall during the flowering season. *Fusarium* infections of cereals lead to severe yield loss and reduced kernel quality in addition to the occurrence of toxins (Glenn, 2007, Ileana Nichita, 2007).

DON is a potent feed intake inhibitor and emetic factor, causing decreased feed intake and weight gain in all evaluated animal species, the most susceptible being pigs and the the most resistant chickens and ruminants (D'Mello, 1999, Pestka, 2007).

Nivalenol (NIV) is mainly produced by *Fusarium cerealis* and *F. poae* but isolates of *F. culmorum* and *F. graminearum* are also able to produce nivalenol (Eriksen, 2003, Glenn, 2007). In contrast to DON, NIV occurs more frequently in years with dry and warm growing seasons. Nivalenol is more common in Europe, Australia and Asia than in America, where the occurrence of NIV is limited. Both mean levels and incidence of positive samples of NIV are lower than for DON even in the Nordic countries and Europe. NIV may occur together with fusarenon X (Fus X), the C-4 acetylated derivative of NIV, and other toxins produced by *Fusarium* fungi (Eriksen, 2003).

T-2 toxin and its deacetylated form, **HT-2 toxin**, normally occur together in cereals. The toxins are mainly produced by *F. sporotrichioides*, but other species have also been shown to produce T-2 toxin and HT-2 toxin. The occurrence of *F. sporotrichioides* in cereals

is mainly a result of water damage to grains occurring when the cereals remain for extended periods on the field at or after harvest or when the grain is wet during storage (JECFA, 2001). According to JECFA (2001), is it not possible to draw any conclusions about what climate is associated with increased levels of T-2 and HT- 2 toxins in cereals, probably due to the differences in optimal growth conditions for the T-2 and HT-2 toxin producing species (JECFA, 2001).

2. BIOSYNTHESIS

The trichothecene biosynthetic pathway in *Fusarium* species begins with a sesquiterpene cyclization catalized by the enzyme trichodiene synthase, followed by up to eight oxygenations and four esterifications. Trichothecene biosynthesis also requires expression of a transporter protein and a network of regulatory genes. Trichothecene biosynthetic and regulatory genes have been mapped to four unlinked loci in the *F. graminearum* genome and also have been localized to specific contigs of the *F. graminearum* genome sequence (Desjardins and Proctor, 2007).

The solubility of trichothecenes varies with the number of polar groups. Most trichothecenes are soluble in solvents like acetone, chloroform and ethylacetate, but highly hydroxylated trichothecenes like DON and NIV are also soluble in more polar solvents like acetonitrile, methanol, ethanol and water (Ueno, 1987 quoted by Eriksen, 2003).

The trichothecene skeleton is chemically stable and the 12,13 epoxide ring is stable to nucleophilic attacks. Furthermore, trichothecenes are heat stable and are not degraded during normal food processing. Trichothecenes are also stable at neutral and acidic pH and are consequently not hydrolysed in the stomach after ingestion (Ueno, 1987 quoted by Eriksen, 2003).

3. TOXIC EFFECTS

Trichothecenes are toxic to all tested animal species, but the sensitivity varies considerably between species and also between the different trichothecenes.

The 12, 13 – epoxide ring is considered to be essential for the toxicity of trichothecenes. In the rat skin irritation assay the de-epoxi T-2 toxin was 400 times less toxic than T2 toxin (Eriksen, 2003).

Most trichothecenes also have a C-9, 10 double bond who is also important for the toxicity. Hydrolisation of the C-9, 10 double bond decreases. Type A trichothecenes, having a functional group other than

carbonyl in the C-8 position, are generally more toxic than B type trichothecenes with a carbonyl in this position (Eriksen, 2003).

In **animals**, the main route of exposure to trichothecenes is ingestion of animal feed of plant origin. T-2 toxin and DAS, which are the most potent for laboratory animals of the trichothecenes commonly reported as feed contaminants (T-2 toxin, DAS, NIV, and DON), induce a similar toxic response. NIV is less potent in some systems than the previous two compounds and DON is the least toxic of the four (examples of potency include the oral LD50s in the mouse: T-2 toxin, 10.5 mg/kg body weight and DON, 46.0 mg/kg) (EHC 105, 1990).

Experimentally, low to moderate dose in acute oral exposure to trichothecenes cause vomiting, diarrhea and gastroenteritis, whereas higher doses cause severe damage to the lymphoid and epithelial cells of the gastrointestinal mucosa resulting in hemorrhage, endotoxemia and shock. Interestingly, this kind of effects can occur in animals exposed to trichothecenes via inhalation. Other targets include bone marrow and thymus which can contribute to generalized immunossuppression (Pestka, 2007).

The most prominent common effects of T-2 toxin, HT-2 toxin, DON and nivalenol at the biochemical and cellular level are: the strong inhibitory effect on the protein synthesis by binding to the ribosomes, the inhibitory effect on RNA and DNA synthesis and toxic effects on cell membranes (SCF, 2002).

Another common effect is the induction of apoptosis particularly in lymphatic and haematopoietic tissue. It appears that different trichothecenes differ in their capacity to inhibit protein synthesis, to activate the mitogen activated protein kinases (MAP kinases) and to induce apoptosis. It is not clear whether the toxins work via identical mechanisms at the biochemical and cellular level (SCF, 2002).

In **humans** the main route of exposure to trichothecenes is also ingestion of contaminated foods of plant origin, but other routes have been reported occasionally, such as accidental skin contact amongst laboratory research workers, and airborne trichothecenes in dust (Peraica, 1999, Sherif, 2009).

There were two disease outbreaks, one reported from China and another from India, where trichothecenes were supposed to have causative role. The outbreak reported from China was associated with the consumption of scabby wheat containing 1.0-40.0 mg DON/kg. The disease was characterized by gastrointestinal symptoms. No deaths occurred in human beings. Swine and chickens fed the leftover cereals were also affected. Another outbreak was reported from India and was

associated with consumption of baked bread made from contaminated wheat. The disease was characterized by gastrointestinal symptoms and throat irritation, which developed within 15 minutes to one hour following ingestion of the bread (EHC 105, 1990, Peraica, 1999).

Two diseases of historical interest, alimentary toxic aleukia (ATA) in the former USSR and scabby wheat toxicosis in Japan and Korea, have been associated with the consumption of grain invaded by Fusarium moulds (Peraica, 1999, JECFA, 2001).

Trichothecenes were found in air samples collected during the drying and milling processes on farms, in the ventilation systems of private houses and office buildings, and on the walls of houses with high humidity. There are some reports showing trichothecene involvement in the development of "sick building syndrome". The symptoms of airborne toxicosis disappeared when the buildings and ventilation systems were thoroughly cleaned (Peraica, 1999).

There are some reports that indicate that trichothecenes may have been used as chemical warefare agents in South-East Asia (Lao People's Democratic Republic and Cambodia) (Peraica, 1999).

4. METABOLISM

Metabolic studies have been carried out on animals, principally with T-2 toxin, but a few with DON. These trichothecenes are rapidly absorbed from the alimentary tract, but quantitative data are not available. The toxins are distributed fairly evenly without marked accumulation in any specific organ or tissue. Trichothecenes are metabolically transformed to less toxic metabolites by such reactions as hydrolysis, hydroxylation, de-epoxidation, and glucuronidation. Trichothecenes, such as T-2 toxin and DON, are rapidly eliminated in the faeces and urine. Only compound traces can still be detected 24 hours after oral exposure (Eriksen, 2003, Pestka, 2007).

5. ANALYTICAL METHODS

Analytical methods based on TLC, GC, HPLC, and immunological techniques are available for the determination of the four most frequently encountered toxins (DON, T-2 toxin, DAS, NIV) with detection limits below 1 μg/g. Several of these methods have been tested collaboratively. In addition, research methods, such as GC/MS and LC/MS, are available for confirmation of identity (EHC 105, 1990, Simona Oancea şi Mihaela Stoia, 2008).

6. TOLERABLE DAILY INTAKE (TDI)

In 2002 the Scientific Committee on Food hade done a group evaluation of T-2 toxin, HT-2 toxin, NIV and DON and established a full TDI for DON and confirmed the temporary TDI (t-TDI) for nivalenol and the combined t-TDI for T-2 toxin and HT-2 toxin:

- DON: TDI = 1 µg/kg bw/day;
- Nivalenol: t-TDI = 0.7 µg/kg bw/day;
- T-2 toxin and HT-2 toxin: combined t-TDI = 0.06 µg/kg bw/day (SCF, 2002).

7. MAXIMUM LEVELS (MLS)

The Maximum Levels for trichothecenes in foodstuffs are set by the Commission Regulation (EC) No 1881/2006 of 19 December 2006, where Deoxynivalenol's MLs vary from 200µg/kg for processed cereal-based foods and baby foods for infants and young children up to 1750µg/kg for unprocessed durum wheat and oats and unprocessed maize. T-2 and HT-2 toxins are regulated as a sum (T-2 and HT-2 toxin) in unprocessed cereals and cereal products (EC No 1881/2006).

8. CONCLUSIONS

8.1. Trichothecenes are a group of sesquiterpenoid mycotoxins produced by feed and food-borne fungi;

8.2. Trichothecenes are toxic for actively dividing cells and they have been associated either with impairment of protein synthesis by binding to the ribosomes of eukaryotic cells, or with the dysfunction of cellular membranes;

8.3. From the large number of trichotecene compounds in this paper were reviewed the ones frequently detected in food and feed that pose a risk to animal and human health: DON, NIV and T-2 toxin.

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SUCCESSFUL OUTCOME AFTER TREATMENT OF A CLINICAL CASE OF VISCERAL LEISHMANIASIS IN A DOG

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Key Words: leishmaniasis, dog, Glucantime, Allopurinol

Summary

A clinical case of visceral leishmaniasis was described in a 4-year old guard dog from Petrich. The diagnosis, visceral leishmaniasis was made on the basis of clinical signs, the epizootiological status of the region and the performed haematological, biochemical and serological analyses. A treatment with Glucantime and Allopurinol was performed according to a schedule and etiotropic therapy was assessed as successful. Some controversial issues with regard to the treatment of *Leishmania infantum* infection of public health importance are discussed.

Visceral leishmaniasis is frequently encountered zoonosis in the countries from the Mediterranean basin. Its seroprevalence reaches 8.5% in Portugal (Alvar, 2001), 1.7-10.0% in Cyprus (Deplazes et al., 1998), 3.6–15% in Israel (Strauss-Ayali et al., 2001), 18.7% in Portugal (Cardoso et al., 2004), 22.1–30.3% in Italy (Zaffaroni et al., 1999), 3–35% in Spain (Sarasa et al., 2000), 3,7–38,8% in Greece (Garifallou et al., 1989; Sideris et al., 1999; Boutsini and Patakakis, 2001), 10–40 % in France (Carlotti, 2003), 42.85% in Croatia (Zivicnjak et al, 2005), 28.9-52% in Malta (Fioretti et al., 1998) and 65–76% in Turkey (Ozbel et al., 2000).

In 2006, the disease is reported for the first time in Bulgaria in dogs from the region of Petrich, and thereafter, in other regions of the country (Tsachev, 2007, Tsachev 2009).

The treatment of leishmaniasis is especially difficult – when delayed, the therapy is often unsuccessful (and in most cases, it does not begin on time); in some countries as Bulgaria there are no specific etiotropic preparations; in some countries (Greece), the therapy is prohibited by the law (because of the impossibility for complete healing and therefore the creation of reservoirs of infection); the occurring resistance to some preparations (antimony derivatives) etc. By now, the therapeutic protocol of leishmaniasis includes different schedules of etiotropic drugs Glucantime, Pentostam, Allopurinol, Pentamidine, Amphotericine B, Paromomycin, Miltefosine (Gramiccia et al., 1992;

Banet, 2001; Lindsay et al., 2002; Miro, 2008; Tsachev, 2009). All they are reported to act with variable success.

MATERIAL AND METHODS

A 4-year female guard dog named Bertha, Rottweiler/German Shepherd cross, weighing 30 kg from Petrich with a regular vaccination history against canine distemper, hepatitis, parvovirosis, parainfluenza, leptospirosis and rabies is described.

Five ml blood was sampled from vena cephalica antebrachii in a vaccutainer with EDTA as anticoagulant and another 5 ml for blood serum collection.

The following blood parameters were assayed: WBC x 10^9/l; RBC x 10^{12}/l; HGB, g/l; HCT, %; MCV, fl; MCH, Pg; MCHC, g/l; PLT x 10^9/l; total protein (g/l), albumin (g/l), albumin/globulin ratio (A/G), urea (mmol/l), creatinine (μmol/l), bilirubin (μmol/l), AP (U/l, ALAT (U/l), ASAT (U/l). The absolute neutrophil, lymphocyte, eosinophil, basophil and monocyte counts were calculated. Haematological studies were performed on an automated counter BC–2800 Vet Auto Hematology Analyzer, Mindray, Korea. Blood biochemical analyses were done with diagnostic kits of Roche Diagnostics GmbH, Germany on a biochemistry analyzer BA–88 Mindrey, Korea.

A serological ELISA test for detection of antibodies against *Leishmania infantum* (IDEXX Snap® Leishmania Test, Maine, USA) was also performed.

RESULTS

The patient's history revealed that six months ago the dog had several episodes of epistaxis. It was treated with doxycycline, prednisolone, dicynone, vitamin C, calcium and glucose. The clinical signs have been controlled, but after two months, a lameness with the left hindlimb has appeared as well as pyometra (that was surgically treated). After the operation, exfoliative dermatitis and alopecia on paws, ears and the nose occurred.

The clinical examination established a variable appetite, bilaterally enlarged popliteal lymph nodes, alopecia on ears, ulcerations on forelimbs and exfoliative dermatitis (Fig. 1). The body temperature of the dog was 39.6 ^oC. The appetite was significantly reduced.

Data from haematological and blood biochemical analysis (Table 1) revealed a marked pancytopaenia – erythrocyte count 4.94×10^{12}/l, total

leukocyte count 4.1×10^9/l and platelets 106×10^9/l. A neutro-lympho-mono-eosinopenia was also present. The blood total protein and globulin levels were increased, as well as activities of enzymes AP, ASAT and ALAT.

The results of the serological ELISA test was positive for *Leishmania infantum*.

The prescribed therapy consisted of Glucantime 75 mg/kg s.c. and Allopurinol 10 mg/kg orally. The duration of the treatment was 28 days.

Fig 1. Visceral leishmaniasis - before (cutaneous lesions) and after treatment

DISCUSSION

The clinical picture (exfoliative dermatitis, variable appetite, enlarged popliteal lymph nodes, auricular alopecia and forelimb ulcerations), the epizootic features (the Petrich region is endemic for autochthonic leishmaniasis among dogs and men), the data from blood laboratory tests (erythropenia, leukopenia, thrombocytopenia and especially the positive result of the serological test) contributed to the diagnostic algorithm of visceral leishmaniasis. The increased blood total protein and globulins are typical for leishmaniasis, as well as higher activities of AP, ASAT and ALAT (Koutinas et al.,1996; Slappendel, 1998; Ciaramella et al., 1997).

The treatment included two of the most commonly used preparations – Glucantime and Allopurinol in combination. The duration of the medical therapy (28 days) is standard and the obtained clinical results: (full growth of hair at alopecia sites, healing of skin lesions, lack of exfoliative dermatitis, regained appetite, normal temperature, normal popliteal lymph nodes), laboratory results (normalization of haematological and blood biochemistry results; Table 1) evidenced the adequately performed treatment.

Hematology and Biochemistry results	Before treatment	After treatment	Ref. Val.*
RBC x 10^{12}/l	4.94	6.68	5.5 - 8,5
HGB x g/l	122	132	120 - 180
HCT %	33.7	45.6	37 - 55
MCV fl	68	68.4	60 - 77
MCH pg -	24.6	26	19 – 23
MCHC g/l	361	381	320-360
PLT x 10^{9}/l	106	358	150 - 500
WBC x 10^{9}/l	4.1	9.5	6·0 - 17·0
NS x 10^{9}/l	2.13	4.65	3·0 -11·5
LY x 10^{9}/l	1.72	3.8	1·0 - 4·0
EO x 10^{9}/l	0	0.66	0·1 - 1·25
MON x 10^{9}/l	0.24	0.38	0·1 - 1·35
BA x 10^{9}/l	0	0	0 - 0.1
Total protein (g/l)	84	62	54 -75
Albumin (g/l)	17	28	25 - 45
Globulin (g/l)	67	34	27 - 44
Albumin/Globulin Ratio (g/l)	0.253	1.21	0.6 – 1.1
Urea (mmol/l)	3.46	6.2	3·3 – 8·3
Creatinine (μmol/l)	116.3	80.3	35 - 106
AP(U/l)	87	67	to 108
ALAT (U/l)	78	-	to 55
ASAT (U/l)	145	41	to 25

Table 1. Hematology and biochemistry results in a dog with visceral leishmaniasis (before and after treatment)
***Ref. Val. = reference values (Kraft and Durr, 1995; Feldman et al., 2000)**

Ten month after this therapy, the performed physical re-examination did not establish any clinical deviations.

The question about the appropriateness for starting a treatment in animals remains still open, because very often, the clinical recovery is accompanied with leishmania carriership (Roze, 2003).

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INFLUENCE OF ENDOMETRITIS ON REPRODUCTIVE PARAMETERS AND FERTILITY ON COWS

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Key words: endometritis, calvinng interval, conception rate.

SUMMARY

After feding, fertility is considereded the factor with the largest economic effect on dairy farms.. Reproductive performance is one key component of dairy production management. Obtaining the reproductive performance requires the mentainance of the parameters at a constant high level Diagnosis and treatment of postpartum uterine disease and its putative impact on reproductive performance, have traditionally attracted considerable attention from veterinarians and producers. The porpose of this study is to evaluate the consequences of endometritis on reproductive parameters and cows fertilitys..Researches conducted on six lots of animals, formed depending on the number of treatments and type of endometritis had the folowing results: there were no differences between healty cows and those with a single treatment, the cows with more treatments had reproductive parameters with large deviationsrelative to the optimal (services per conception +0,83; first-service conception rate -12,6%; days open +40,9; calving-first insemination interval +14,2 days), endometritis type (E4, E3, E2) change in this order intervals calving-first insemination, last treatment-first insemination, last treatment-conception, days open, services per conception). Cows with endometritis type 4 (result of retained placenta) presents the largest deviation: services per conception 2,77 ;days open 118,3 and the last treatment-conception interval 61,3 days, folowed by those with endometritis, type 3 and 2. Endometritis causes anestrus blocking lysis of the corpus luteum at 21,5 from animals (the lot X1), 5,2%(lot X1) face of 6,3% in the lot M (healty animals). Between the infertility factors, endometritis represented the highest percentage(3%), folowed by vaginal inversion and parametritis.

Obtaining and maintaining a good reproductive performance is the key in to the management of dairy farms in our days. After feeding, fertility is considered the largest economic factor of the dairy farms. Among the common uterine disorders during puerperal period, endometritis plays the most important role.

After parturition the uterine lumen, considered sterile, is contaminated by bacteria from the environment, the period of maximum risk being 0-24 days postpartum. Intensively managed herds of dairy cattle often have uterine bacterial contamination rates of 90 to 100% within the first two weeks post partum. Endometritis results from a variety of factors favoring and determinants. Factors which favoring are dystocias, remedial interventions, retained placenta, uterine subinvolution, nutrition deficiency, decreased immunity and hygienic

conditions in stables. Endometritis incidence in dairy farms varies widely, from 10 to 50%, with an average of 22% (Drugociu, 2004) depending on the season and ensuring the specific zooigienis measures in puerperal period.

Several studies had evaluated the causes, incidences and effects of uterine disorders on reproductive performance, establishing that endometritis affect all parameters of long-term, and serious economic effects resulting from the exploitation costs and hormonal treatments performed (cloprostenol between 20-33 DIM-Leblanc et al, 2002).

Everyone agrees primary endometritis role in reducing the reproductive performance: reducing pregnancy rate by 27%, increasing services per conception with 0.35, the service period's with 30 days and the percentage of infertility with 3% (Leblanc et al, 2002). Increased calving-interval duration produce large economic damage estimated at 3€ for each day of delay after the 365 (M. Benzaquen et al, 2007). Cows with endometritis had a lower conception rate (33%) versus 53% in healthy cows; they need more inseminations for pregnancy (2.6 to 1.8) and a longer calving-interval with 35 days (R.W. Blowey, 2000).

Exploitation of animals with high economic value and use of latest generation tehnology in dairy farms, requires a maximum economic efficiency, achieved by maintaining reproductive parameters in a high level.

The purpose of this paper is to assess the impact of endometritis, part of uterine disorders, on reproductive parameters and fertility in a dairy farm.

MATERIAL AND METHOD

The study was conducted from January 1, 2009 to May 31, 2009 on a total of 190 cows, breed Bălțată românească, maintained in loose housing and monitored through a computerized management system of the type AfiFarm.

The research has covered the pathology of puerperal period, particularly endometritis detection, forming six lots of animals according to the following criteria:

1. The presence of endometritis and the number of treatments:

- the lot M-clinically healthy cows, without endometritis, without treatments;
- the lot X1-cows with endometritis, which received a single treatment;

- the lot X2-cows with endometritis who received several treatments

2. Type of endometritis:

- the lot E4-cows with liquid discharge, brown, with remnants of uterine placenta and with fetid odor;
- the lot E3-cows with semi leaks discharge with mucus, jelly and fetid odor;
- the lot E2-cows with purulent discharge, with mucus and no umpleasant odor.

In a first phase, we calculated the reproductive parameters (calving-first artificial insemination interval, days open, services per conception, calving interval, conception rate and gestation index) for lots M, X1 and X2. For this porpose we used statistics recorded in AfiFarm program for each of 190 animals (all treatments and interventions for each animal, type of endometritis, number and date of artificial insemination, age at first insemination). We compared values of reproductive parameters calculated for each lots (M, X1, X2) and analyzed results.

For lots E4, E3, E2 we calculated the following parameters: last treatment-first artificial insemination period, the time last treatment - artificial insemination fertile, period parturition-first artificial insemination, service period, insemination index).

To determine the role of endometritis in producing and maintaining anestrus on cows (considered as no event heats up to 55 days post-partum) we calculated the percentage of cows without heats in lot M (control group, cows without endometritis), X1 and X2 (cows with varying degrees of endometritis).

2. RESULTS AND DISCUSSION

Research conducted on lots M, X1 and X2 (table 1), consisted of determining the reproductive's parameters for each of them. The results showed that the cows without endometritis had the best reproductive's parameters (70% conception rate, pregnancy rate 89%, insemination index 1.6, service-period 64.7 days, time parturition-first artificial insemination 58.2 days).

Table 1

EXPERIMENTAL GROUPS

	LOT			TOTAL
	M (without treatment)	**X1 (with a single treatment)**	**X2 (with several treatments)**	
n	47	38	105	190
%	25%	20%	55%	100%

Cows that required a single treatment had reproductive parameters similar with healthy cows, demonstrating that light endometritis detected early and treated appropriately didn't have any negative effect against reproductive parameters values and fertility.

Cows that received two or more treatments had reproductive parameters with large deviations to the optimal (insemination index was higher 0.83 (2.43), lower conception rate by 33% (44.1%), pregnancy rate was lower 12.6% (76.4%), service period's higher 40.9 days (107.6), calving interval greater than the 40.9 days (392.6) and parturition-first artificial insemination period more with 14.2 days (table 2).

Table 2

REPRODUCTIVE PARAMETERS VALUES DEPENDING ON THE NUMBER OF TREATMENTS

INDICI	LOT		
	M (without treatment)	**X1 (with a single treatment)**	**X2 (with several treatments)**
P-IA	58,2	57,4	75,4
SP	64,7	66,7	107,6
Ii	1,60	1,61	2,43
Rc %	70	68	44,1
G%	89	91	76,4
CI	349,7	351,7	392,6

P-IA- parturition-first artificial insemination interval (days)

SP – service – period (days)

Ii – insemination index (services per conception)

Rc% - conception rate (pregnants after first insemination)

G% - pregnancy rate

CI – calving interval (interval between parturitions)

Presence of endometrial infections has reduced reproduction parameters, number of treatments required for healing was congruent with the seriousness and type of endometritis. The causes were: the long period needed for healing and sterilization of the uterus, the

endometrium recovery and achieve optimal conditions for implantation and pregnancy mostly for animals that received two or more treatments. Although the first cycle after the last treatment animals not shown clinical signs of endometritis, the conception rate was 44.1% (elimination of germs continuing and after absence of clinical signs).

Increcrease of sevices number per conception resulted directly increase number of days open and reduce pregnancy and conception rates.

Research on lots E4, E3, E2, called by endometritis degree included determining intervals: the last treatment-first artificial insemination, - artificial insemination fertile, service-period, services per conception. Calculated parameters varied depending on the endometritis degree: cows with endometritis type 4 had the most ervices per conception (2,77), the largest service-period (118,3 days), the longest period parturition-first insemination (88.7 days) and period last treatment - conception (61.3 days). Parameters values for cows with endometritis type 3 (13.5%) were smaller, the last treatment – first insemination high as 8 days. Cows from lot E2 detected with endometritis, type 2 (69.4%) had the closest parameters to optimal values, with 2,25 services per conception and 91,6 days open (table 3).

Table 3

RELATION BETWEEN ENDOMETRITIS TYPE AND REPRODUCTIVE PARAMETERS

ot		%	last treatment-I I.A. interval	last treatment-conception	parturition I-a I.A.	Servic e-period	Insemin ation index (services per concepti on)
4	4	17,1	31,6	61,3	88,7	118,3	2,77
3	9	13,5	39,7	48,9	74,5	95,7	2,72
2	1	69,4	30,4	50,2	72,1	91,6	2,25

Endometritis type determines the time needed healing animals, heats appearance, the first artificial insemination and conception. Endometritis type 2 had the lowest effect on reproductive parameters, the type 4 being the worst in time of healing and fertility over effects. The following research on lots M, X1 and X2 were taken in the study cows with pathological anestrus (no heats up to 55 days postpartum. Analyzing

graphs, anestrus frequency to animals without endometritis was 6,3%, to those with mild endometritis 5,2% and 21,5% to those with severe endometritis (with multiple treatments).

Table 4

THE EFFECT OF ENDOMETRITIS TO ESTRUS CYCLE

Anestrus 55 zile	Lot		
	M	X1	X2
n	3	2	22
%	6,3	5,2	21,5

Endometritis are blocking the synthesis and release of Pg F2α , lysis of corpus luteum and sexual cycle expression. The results presented in table 4 confirm the correlation between endometritis severity and time necessary to first cycle expression. Research conducted on the entire population showed that endometritis are a leading cause of acquired infertility (3%), with vaginal inversion (2,6%) and parametritis (table 5)

Table 5

ENDOMETRITIS - CAUSE OF INFERTILITY

STERILITY		
	N	%
parametritis	2	1,1
perineal rupture	1	0,5
vaginal inversion	5	2,6
uterine prolapse	1	0,5
endometritis	6	3
TOTAL	15	7,8

Endometritis caused infertility by artificial insemination failure (chronic endometritis presence) and by creating adverse conditions for viability and migration of sperm and zygote. Second, endometrial lining defacement and salpingitis are sterility factors for cows with endometritis

3.CONCLUSIONS

3.1 There were no significant differences between healty cows (from lot M) and those with a single treatment (from lot X1).

3.2 Cows with more treatments had reproductive parameters with large deviations relative to the optimal (services per conception +0,83

(2,43), conception rate -33% (44,1%); pregnancy rate -12,6% (76,4%); days open +40,9 days (107,6); calving interval, +40,9 days (392,6);

3.3 Endometritis type (E4, E3, E2) change in this order the reproductive parameters values: cows with endometritis type 4 had large deviations to the optimal values: last treatment-first insemination, last treatment-conception (61,1 days), days open (118,3), services per conception (2,77);

3.4 21,5% of cows from group X2 (with multiple treatments) showed no heat up to 55 days compared to 5,2% (group X1) and 6.3% (group M), and are more likely to anestrus;

3.5 Endometritis causes sterility to 3% of the herd and represents 38% of cases of infertility.

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THE INCIDENCE OF AFLATOXIN B_1 AND OCHRATOXIN A IN NON-ANIMAL PRODUCTS IN THE COUNTIES FROM THE WEST AREA OF ROMANIA IN THE PERIOD 2007-2008

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Keywords: aflatoxin B_1, ochratoxin A, peanuts, nuts, dried fruits

SUMMARY

Between 2007 and 2008 in the mycotoxins laboratory from the Sanitary Veterinary and Food Safety Directorate Timiş, 350 samples were analyzed for aflatoxin B1 and ochratoxin A: 40 samples of peanuts, 36 pistachios, 95 nuts, 58 dried fruits, 45 roasted coffee, 38 ground coffee and 38 fruit juices. The study revealed that: there were no positive samples no positive samples of peanuts, pistachios, fruit juices and dried fruits in 172 analyzed samples; 15.8% of nuts contaminated with AFB1, not surpassing of maximum permitted level (MPL); 8.9% of roasted coffee and 5.6% of ground coffee were contaminated with OTA, but no positive sample was over MPL; the samples of dried fruits were analyzed for the presence of both AFB1 and OTA and no sample was cross contaminated.

Many people consider natural products to be safe. Dried fruits, raisins, peanuts or ground nuts are good substrate for the toxinogenic moulds to develop. Their toxins and metabolites can be in direct contact with human consumers, affecting their health (Bărzoi et al., 1999).

The FAO/WHO experts discussed diverse hazards that present human health risks in their Report after the 2007 annual meeting, the main subject being animal feed impact on food safety (FAO/WHO, 2007).

Aflatoxin contamination is not homogenous and this is the reason of using special methods for selecting the samples (Wilm, 2005). Romanian legislation has adapted these special methods from the European legislation and every detail is described, including the necessary quantities for raw materials and the analytical methods used (Romanian legislation).

The scope of this research was to evaluate the frequency of mycotoxin contamination of different categories of products of non-animal origins in the West counties of Romania, during 2007-2008 period.

The study had as objectives to emphasize:

- The frequency of aflatoxin B_1 (AFB_1) and/or ochratoxin A (OTA) contamination of ground nuts, dried fruits, peanuts, pistachios, nuts, coffee and fruit juices;
- The contamination levels with AFB_1 and/or OTA of the products of non-animal originins.

MATERIALS AND METHODS

In the mycotoxin laboratory, part of Sanitary Veterinary and Food Safety Directorate of Timis county, between 2007-2008 were analyzed 350 samples for mycotoxin contamination. The samples were collected from the West region – Arad, Bihor, Caras Severin, Mehedinti, Satu Mare and Timis counties: 40 samples of peanuts, 36 pistachios, 95 nuts, 58 dried fruits, 45 roasted coffee, 38 ground coffee and 38 fruit juices.

For the dosage of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) in these products, ELISA methods were used. Kits were produced by R-Biopharm or Eurodiagnostica. Methods are accredited and validated. The technicians from the laboratory participated to proficiency tests like FAPAS with satisfactory results.

RESULTS AND DISCUTIONS

From 134 analyzed samples of peanuts, pistachios and dried fruits no positive sample were detected, as presented in Table1**.**

Table 1

Frequency of samples analyzed for contamination with AFB_1

County	Types of samples					
	Peanuts		Pistachios		Dried fruits	
	Analyzed samples	positive samples	Analyzed samples	positive samples	Analyzed samples	positive samples
AR	10	0	7	0	9	0
BH	8	0	7	0	4	0
CS	5	0	3	0	4	0
MH	5	0	3	0	5	0
SM	4	0	7	0	5	0
TM	8	0	9	0	36	0
Total	40	0	36	0	58	0

From a total of 95 samples of nuts, 15 (15.8%) were found positive for AFB_1. The most frequent positive samples were found in Timis county (22.8%) and Arad county (20.0%), and lower levels of contamination were found in Satu Mare (12.5%) and Caraş Severin

county (9.1%). In Bihor, and Mehedinți county no positive samples were found.

The level of contamination presented in Table 2 shows that no sample exceeded the maximum permitted level.

Table 2

Frequency and mean level of AFB_1 (ppb) in nuts

County	Analyzed samples	Positive samples		Values			
		No	%	Minimum	Maximum	Mean	Median
AR	25	5	20.0	0.527	1.35	0.720	0.715
BH	9	0	0	0	0	0	0
CS	11	1	9.1	0	1.22	0	0
MH	7	0	0	0	0	0	0
SM	8	1	12.5	0	0.625	0	0
TM	35	8	22.8	0.535	1.44	0.736	0.788
Total	95	15	15.8				

Ochratoxin A was found in 4 samples (8.9%), from a total of 45 analyzed samples of roasted coffee. The most frequent positive samples were detected in Timis county 2, 16.6% from 12 analyzed samples of roasted coffee, followed by Arad 14.3% and Bihor county 12.5%.

The level of contamination with OTA is presented in Table 3.

Table 3

Frequency and mean level of OTA (ppb) in roasted coffee

County	Analyzed samples	Positive samples		Values			
		no	%	Minimum	Maximum	Mean	Median
AR	7	1	14.3		3.15		
BH	8	1	12.5		2.48		
CS	5	0	0	0	0	0	0
MH	7	0	0	0	0	0	0
SM	6	0	0	0	0	0	0
TM	12	2	16.6	1.75	3.35	2.55	
Total	45	4	8.9				

No positive samples for ochratoxin A were found in Caras Severin (five samples), Mehedinti (seven samples) and Satu Mare counties (six samples) for roasted coffee.

From 38 analyzed samples of ground coffee, only two were positive, 5.3% and the level of contamination is presented in Table 4.

Table 4

Frequency and mean level of OTA (ppb) in ground coffee

County	Analyzed samples	Positive samples		Values
		no	%	Maximum
AR	9	1	11.1	3.25
BH	5	0	0	
CS	5	0	0	
MH	3	0	0	
SM	7	0	0	
TM	9	1	11.1	3.75
Tot	38	2	5.3	

Data concerning the frequency of contamination with ochratoxin A of fruit juices and dried fruits in six counties from the West region of Romania is presented in Table 3. No positive samples of dried fruits or fruit juices were found for OTA.

Table 3

Frequency of samples contaminated with OTA

County	Types of samples			
	Fruit juice		Dried fruits	
	Analyzed samples	Positive samples	Analyzed samples	Positive samples
AR	11	0	9	0
BH	5	0	4	0
CS	3	0	4	0
MH	3	0	5	0
SM	9	0	5	0
TM	7	0	36	0
Tot	38	0	58	0

The associated contamination of AFB_1 and OTA was searched in 58 samples of dried fruits, but no sample was positive for cross contamination.

Further monitoring and control is needed for these products as well as for dried vine fruit, grape juice, soluble coffee and beer.

In Austria, aflatoxin contamination in special kinds of imported food sometimes exceeds EU maximum levels. The rate ranges from 14 to 47% depending on the product (14% in peanut, peanut products and 47% in pistachio). In 36 samples of imported coffee analyzed for ochratoxin A, the range was below maximum levels (Ohlinger et al., 2004).

A study carried out in Belgium, on large number of samples, the levl of AFB_1 was below the limit of quantification. However, eight contaminated samples exceeded the EC maximum limit: one sample of Brazil nut (in 2001), two samples of hazelnuts (one in 2001 and one in 2002) and five samples of pistachios (in 2001). The highest level of contamination was found in pistachios (one sample analyzed in 2001 contained 52.5 μg/kg). In 58 samples of dried fruits, coffee and fruit juice analyzed for OTA, the values found were below MP.(Chandelier et al., 2004).

In Cyprus, the highest incidence of aflatoxin contamination observed, was in imported pistachios (10%) for the last years 2002-2003 and the highest level was 785 μg/kg total AFs in peanuts for the year 1997. In contrast to the peanuts and pistachios and most recently Brazil nuts, other nuts (cashew nuts, walnuts, hazelnuts, coconut), dried fruit and other products were almost negative to AFB_1. The levels of OTA in imported (green coffee) ranged between 0.6 and 5.0 μg/kg and were within the proposed EU limit(Ioannou-Kakouri et al., 2004).

In Czech Republic, 432 samples of peanuts were analyzed for AFB_1 and 19 were positive with a maximum value of 161.0 μg/kg, in the period 2000-2002. For OTA were analyzed, also, 11 samples of coffee, but none were non-compliant (Ostry et al., 2004).

Raisins, cereals, coffee, wine, beer, grapes juice, spices, vegetables and some animal tissues may be sources of contamination with ochratoxin A. This toxin is not completely destroyed by technological processes or by cooking (Ostry et al., 2002).

Studies carried out in Germany pointed out that, within a monitoring program developed between 1995 and 1999, about 50 to 70% of pistachio samples contained aflatoxins exceeding the maximum admissible level. Since 1999, the situation has gradually been improved, and less than 20% of samples exceeded the maximum admissible level. Pre-processed material may also be of importance, as demonstrated by the high aflatoxin B_1 contamination of up to 415 μg/kg found in pistachio-paste for ice-cream production. About 50% of roasted and ground coffee samples, and up to 90% of instant coffee samples were found positive for OTA. The mean contamination level was highest in instant coffee with caffeine (1.83 μg/kg). Almost 100% of raisins and currants samples were found OTA positive (maximum 21.4 μg/kg)(Curtui et al., 2004).

In Italy, 21 samples of the major coffee brands distributed at national level were screened and were found 16 of them contaminated with OTA. In particular, the maximum level (13 ng/g) was found in

soluble coffee, and a marked presence of OTA remained in roasted samples(Moretti et al., 2004).

CONCLUSIONS:

The present study, carried out during 2007-2008 period, revealed:

3.1 no positive samples of peanuts, pistachios, fruit juices and dried fruits in 172 analyzed samples;

3.2. that 15.8% of nuts contaminated with AFB_1, not surpassing of maximum permitted level (MPL);

3.3. that 8.9% of roasted coffee and 5.6% of ground coffee were contaminated with OTA, but no positive sample was over MPL;

3.4. the samples of dried fruits were analyzed for the presence of both AFB_1 and OTA and no sample was cross contaminated.

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THE INCIDENCE OF AFLATOXIN M_1 IN MILK AND DIARY PRODUCTS IN THE WEST COUNTIES OF ROMANIA IN THE PERIOD 2004-2008

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Keywords: aflatoxin M_1 (AFM_1), raw milk, milk products

SUMMARY

Between 2004 and 2008 in the mycotoxins laboratory from the Sanitary Veterinary and Food Safety Directorate Timiş, 1322 samples were analyzed for aflatoxin M_1: 815 raw milk, 147 consumption milk, 70 powder milk, 172 yogurts, 58 cheeses, 60 butter samples. The study revealed that: there were no positive samples in 290 analyzed samples of yogurts, cheeses and butter; 15.0% of raw milk samples were contaminated with AFM_1, and 26.2% of the positive samples were over maximum permitted level; lower level than maximum permitted level of contamination in consumption and powder milk was registered.

The FAO/WHO experts discussed diverse hazards that present human health risks in their Report after the 2007 annual meeting, the main subject being animal feed impact on food safety. (FAO/WHO, 2007)

In the last decade, many studies have been conducted on mycotoxins. Most frequently occurring mycotoxins (aflatoxin B_1, ochratoxin A, zearalenone, fumonisin B_1, deoxinivalenol, T-2 and HT-2) are currently considered for their effects on animal health. However, when focusing on how mycotoxins play a role in food safety, attention should be limited to mycotoxins that are known to be transferred from feed to food of animal origin, as this food represents a significant route of exposure for humans. There are well known the transfers from feedstuffs to foodstuffs: in liver is found aflatoxin B_1, in milk aflatoxin M_1, in eggs aflatoxicol, in meat ochratoxin A, in meat DON as DOM_1, zearalenon in meat as zeranol. (Bărzoi et al., 1999, Rankin and Grau, 2003 and Wilm, 2005)

Aflatoxin contamination is not homogenous and this is the reason of using special methods for selecting the samples. Romanian legislation has adapted these special methods from the European legislation and

every detail is described, including the necessary quantities for raw materials and the analytical methods used. (Romanian legislation)

In the 2001-2007 period, studies conducted revealed that the most feedstuffs from the Timis county were contaminated with fungi from Penicillium, Aspergillus and Fusarium species, the main „producers" of aflatoxin B_1 and ochratoxin A. (Trif et al., 2005 and Damiescu and Trif, 2008)

In ruminants, aflatoxin B_1 is metabolized by various enzymes and can be excreted in the milk as aflatoxin M_1 (AFM_1). Because children are the main milk consumers it is important to detect even low levels of AFM_1 in milk and milk products.

Romanian legislation has been adapted to EU legislation and the maximum admitted level for AFM_1 in milk and milk products is 0.05 μg/kg. (Commision Regulation)

The scope of this research was to evaluate the frequency of aflatoxin M_1 contamination of different categories of milk and diary products in the West county of Romania, during 2004-2008 period.

The study had as objectives to emphasize:

- The frequency of aflatoxin M_1 (AFM_1) contamination of raw milk, pasteurized and powder milk and milk products – yogurts, cheeses, butter;
- The contamination levels of AFM_1 in milk and diary products.

MATERIALS AND METHOD

In the mycotoxin laboratory, part of Sanitary Veterinary and Food Safety Directorate of Timis county, between 2004-2008 were analyzed 1322 samples of raw milk and diary products for AFM_1 contamination assessment. The samples were collected from the West region – Arad, Bihor, Caras Severin, Mehedinti, Satu Mare and Timis counties: 815 raw milk, 147 consumption milk, 70 powder milk, 172 yogurts, 58 cheeses, 60 butter samples.

For the dosage of aflatoxin M_1 (AFM_1), ELISA method was used. Kits were produced by R-Biopharm or Eurodiagnostica (protocoale ELISA). The method is accredited and validated. The technicians from the laboratory participated to proficiency tests like FAPAS with satisfactory results.

REZULTS AND DISCUTIONS

Data concerning the frequency of contamination with aflatoxin M_1 of raw milk samples in six counties from the West region of Romania is presented in Table 1.

The milk samples were collected from cow, sheep and buffalo farms and milk producers.

Table 1

Frequency and level of contamination with AFM_1 (ppb) of raw milk samples

County	Total number samples	Positive samples		Samples over MPL		Values			
		no	%	no	%	Minimum	Maximum	Mean	Median
AR	132	19	14.4	7	36.8	0.067	0.275	0.086	0.062
BH	95	12	12.6	3	25	0.063	0.074	0.088	
CS	77	9	11.7	2	22.2	0.075	0.089	0	0
MH	68	7	10.3	0	0	0.025	0.047	0.035	0.029
SM	247	39	15.8	11	28.2	0.064	0.327	0.079	0.085
TM	196	28	14.3	9	32.1	0.058	0.304	0.087	0.094
Total	815	122	15.0	32	26.2				

In raw milk, from a total of 815 samples, 122, respectively 15.0% were found positive for AFM_1. From the positive samples, 32 samples (26.2%) were over the maximum admitted level of 0.05 μg/kg (0.05 ppb).

The most frequent positive samples were found in Satu Mare county (15.8%), Arad (14.4%) and Timis county (14.3%), and the lowest contamination was found in Mehedinți county (10.3%). In the Bihor and Caras Severin counties, the percentages of positive samples were among media, 12.6%, respectively 11.7%. The highest value was 0.327 ppb in Satu Mare county. The quantification level is 0.025 ppb (the limit for a sample to be considered positive).

The frequency of consumption milk, powder milk, yogurts, cheeses and butter contaminated with AFM_1 is presented in Table 2 and Table 3.

Table 2

Frequency of consumption and powder milk contaminated with AFM_1

County	Type of products					
	Consumption milk			Powder milk		
	Analysed samples	Positive samples		Analysed samples	Positive samples	
		no	%		no	%
AR	21	5	23.8	14	3	21.4
BH	22	3	13.6	7	0	0

CS	34	7	20.6	11	2	18.2
MH	9	0	0	5	0	0
SM	33	5	15.2	18	4	22.2
TM	28	4	14.3	15	3	20.0
Total	147	24	16.3	70	12	17.1

Table 3

Frequency of diary products contaminated with aflatoxin M_1

County	Types of products					
	Yogurts		Cheeses		Butter	
	Analysed samples	Positive samples	Analysed samples	Positive samples	Analysed samples	Positive samples
AR	35	14	10	10	10	0
BH	32	8	7	7	7	0
CS	22	9	8	8	8	0
MH	17	5	9	9	9	0
SM	32	11	14	14	14	0
TM	34	11	12	12	12	0
Total	172	58	60	60	60	0

No positive samples were found in 172 yogurt samples, 58 cheeses and 60 butter samples.It is imperative to detect the presence of AFM_1 in baby foodstuffs (1) and in all imported baby powder milk or other diary products.

From 147 consumption milk samples, 24 (16.3%) were positive but the level of contamination was below the maximum permitted level (MPL) of 0.05 ppb (µg/kg), Table 4.

Table 4

Mean level of AFM_1 (ppb) in consumption milk

County	Analysed samples	Positive samples		Values			
		no	%	Minimum	Maximum	Mean	Median
AR	21	5	23.8	0.025	0.048	0.034	0.036
BH	22	3	13.6	0.027	0.044	0.031	0
CS	34	7	20.6	0.025	0.045	0.034	0.029
MH	9	0	0	0	0	0	0
SM	33	5	15.2	0.029	0.045	0.035	0.034
TM	28	4	14.3	0.028	0.048	0.036	0.033
Total	147	24	16.3				

Only 12 powder milk samples (17.1%) were positive from a total of 70 analyzed samples. The level was lower than MPL, Table 5.

Table 5

Mean level of AFM_1 (ppb) in powder milk

County	Analysed samples	Positive samples		Values			
		no	%	Minimum	Maximum	Mean	Median
AR	14	3	21.4	0.027	0.044	0.035	0.035
BH	7	0	0	0	0	0	0
CS	11	2	18.2	0.031	0.047	0	0
MH	5	0	0	0	0	0	0
SM	18	4	22.2	0.026	0.045	0.036	0.033
TM	15	3	20.0	0.027	0.048	0.037	0
Total	70	12	17.1				

More than 1300 samples were analyzed in Belgium over two years (2001-2002) and only one raw milk sample was non-compliant. Some samples of powdered milk (20%) also exceeded 0.050 µg/kg (with ranging from 0.050 and 0.115 µg/kg), but the water content must be taken in account. No sample was rejected. (Chandelier et al., 2004)

Between 1995-2003, in Cyprus over 289 raw milk samples (cow, goat, sheep) and pasteurized milk (cow) were analyzed and were not only below the Cyprus ML (0,5 µg/L), but well below the lower ML(0,05 µg/L) adopted by the EU legislation. A higher number of positive milk samples were observed in raw compared to pasteurized milk. Not one positive sample was observed in imported baby milk. The authors consider that the control must be expanded to cover all the types of baby food which contain milk, the milk powders used in dairy and other food industries and diary products such as local and imported cheeses, yogurts and whey cheeses. (Ioannou-Kakouri et al., 2004)

In Czech Republic, between 2000-2002, more than 144 raw milk samples and milk based products were analyzed. Only one positive sample was found, containing 0,5 µg/kg. (Ostry et al., 2004)

The control of imported feedstuffs for aflatoxin B_1 resulted in a marked decrease of aflatoxin M_1 (AFM_1) levels in milk. In the late 1980s, domestic milk contaminated with AFM_1 was relatively often identified, the levels occasionally exceeded the maximum admissible level (0.050 µg/kg). Due to stringent control of certain imported feedstuffs, the situation has been changed in the 1990s, when the incidence of AFM_1 in milk decreased yearly. Continuing monitoring studies of farm milk for AFM_1 performed in several states of Germany in recent years pointed out that – in almost all samples – toxin levels were below 0.010 µg/l. (Curtui et al., 2004)

In 2003, an extraordinary level of AFB_1 contamination of maize-based feeds in the Northern Italy caused also the highest incidence of

milk contamination by AFM_1 recorded during the last decade. Data provided by Pietri, quoted by Moretti pointed out that AFM_1 was over the low limit in around 50-60% samples investigated in October 2003. Although this percentage decreased in further analysis performed, the percentage of highly contaminated samples by AFM_1 still has been constantly high during the following five months. (Moretti et al., 2004)

Between 1999-2001, more than 68 raw milk samples were analysed in Portugal. 60 samples were positive for AFM_1, but with levels of contamination between 0.010-0.024 μg/L. (Peito and Venancio, 2004)

CONCLUSIONS:

The present study, carried out during 2004-2008 period, in the West region in milk and diary products, revealed:

3.1. the contamination with AFM_1 of 15.0% of raw milk samples;

3.2. surpassing of maximum permitted level in 26.2% of positive samples for AFM_1 in raw milk;

3.3. lower level than the maximum permitted level of contamination with AFM_1 of consumption and powder milk;

3.4. absence of AFM_1 in 290 samples of yogurts, cheeses and butter.

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A CLINICAL CASE OF TOXOPLASMIC ENCEPHALITIS IN A CAT

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Key words: toxoplasmosis, encephalitis, seizures, tetraparesis

SUMMARY

The authors reveal clinical aspects of encephalitis in an young adult cat presented in the Internal Medicine Department of The Faculty of Veterinary Medicine Bucharest. It can be pointed the peracute onset of neurological clinical signs that rapidly progresses. The cat benefit from an acurate physical examination, ultrasound evaluation, hemogram and serum chemistry determinations. The imaging findings of the MRI that determined the portion of the brain most affected by an encephalitic process, in concordance with the clinical signs, and the positive result of the serologic test of anti-toxoplasmic antibodies estabished the final diagnosis. The patient responded to the specific terapy for toxoplasmosis and that confirm the diagnosis.

Toxoplasmosis is a disease caused by a single-celled parasite called *Toxoplasma gondii* (T. gondii). Toxoplasmosis is one of the most common parasitic diseases and has been found in nearly all warm-blooded animals, including pets and humans. Despite the high prevalence of *T. gondii* infection, the parasite rarely causes significant clinical disease in cats-or any species. Cats, both wild and domestic, are the only definitive hosts for *Toxoplasma gondii*. This means that the parasite can only produce oocysts (eggs) when infecting a cat. Other animals, including humans, are intermediate hosts of *Toxoplasma gondii*. These hosts can become infected but do not produce oocysts. The most common symptoms of toxoplasmosis include fever, loss of appetite, and lethargy. Other symptoms may occur depending on whether the infection is acute or chronic, and where the parasite is localized in the body: pneumonia (lungs), edema of the retina (posterior chamber of the eye), abnormal pupil size and responsiveness to light (anterior ocular chamber), blindness, incoordination, heightened sensitivity to touch, personality changes, circling, head pressing, twitching of the ears, difficulty in chewing and swallowing food, seizures, and loss of control over urination and defecation (central nervous system).

Toxoplasmosis is not easy to diagnose. The diagnosis is based on the history, signs of illness, and the results of supportive laboratory

tests: serology's values of IgG and IgM antibodies to *Toxoplasma gondii*. In acute onset of the disease, specific antitoxoplasmic treatment is started immediately. If clinical improvement is not seen within two to three days, the diagnosis of toxoplasmosis should be questioned.

1. MATERIAL AND METHOD

The study has been done on one feline presented to the Internal Medicine Clinic Department of the Faculty of Veterinary Medicine Bucharest and whose clinical findings oriented the diagnose to encephalitis.

For precising the diagnose, the patient followed the next protocol: anamnesis, physical examination, hemathology and serum chemistry data, imaging examination – thorax RX and abdominal ultrasound, ophalmologic examination and serum test of IgM and IgG antibodies to Toxoplasma gondii. The most valuable data were obtained from the clinical investigation and ophtalmological findings. The final diagnosis was made coroborating the serum concentration value of IgM antibodies to *Toxoplasma gondii* with the imaging MRI findings, which was acute toxoplasmic encephalitis.

2. RESULTS AND DISCUTIONS

Physical examination revealed normal corporal temperature, mucosa and respiratory and cardiology findings, but the neurological examination give us the most important clinical data. The cat had petit mal type cluster crises in the last 24 hours. At the moment of examination the patient had cluster seizures which last for one and a half hour before responding to terapy and after that, circling, diminished proprioceptive sensibility and motor deficit on the right side. In the next 7 days the cat manifests severe encephalitic signs: non-responsive status and paraparesis.

The ophalmologic examination results from the first day were: positive pupiplary reflex and optic nerv papilary edema. Thoracic and abdominal imaging had normal results. Serum chemistry profile was in normal ranges and the hematology result was mild leukocytosis. The MRI results were very important for the final diagnosis. The serum test rezults (high IgM and low IgG) for anti-toxoplasma antibodies give us the final diagnosis. Differential diagnosis included the usual 7 steps: vascular (brain ischemia or heamoragia), infectious (mycotic – cryptococcosis, blastomycosis; bacterial; rickettsial disease; viral –

rabies, FIP; parasitic – toxoplasmosis, dirofilariosis), metabolic or toxic, immune-mediated (FIV, FeLV are risk factors), central nervous sistem neoplasia, degenerative, idiopathic.

The diagnosis was confirmed by the recent history of the patient (consumer of raw beef meat from an unaviced source) and by the positive and rapid response to the specific terapy (clindamycin for 10 days followed by pyrimethamine and sulfadiazine for another 10 days). After 20 days of treatment the cat was completely recovered, without neurological sequele and the serum antibodies test confirmed a recent infection with Toxoplasma (high IgG and IgM within the range).

The owner was a pregnant women int he last 1/3 of pregancy. She was tested for toxoplasmosis and the result was negative for acute diseases and well protected against the disease (high IgG titre). The gynecologist doctor concluded that it was no risk for the child.

It has to be pointed the way in which this disease occur in cats and how big is the potential danger for the humans they share the living with. The clinical disease-toxoplasmosis-occurs when the cat's immune response is not adequate to stop the spread of tachyzoite forms: in cats with suppressed immune systems, including young kittens and cats with feline leukemia virus (FELV) or feline immunodeficiency virus (FIV). During the intraintestinal infection cycle in the cat, some T. gondii organisms released from the ingested cysts penetrate more deeply into the wall of the intestine and multiply as tachyzoite forms. These forms then spread out from the intestine to other parts of the cat's body, starting the extraintestinal infection cycle. Eventually, the cat's immune system restrains this stage of the organism, which then enters a dormant or "resting" stage by forming cysts in muscles and brain. These cysts contain bradyzoites, or slowly multiplying organisms.

Humans have a lot of sourses of contamination with *Toxoplasma gondii* except from cat feaces and they are usually well protected against this disease by high IgG serum titres. The contamination sources are: oocyst-contaminated soil, undercooked infected meat, particularly lamb and pork, unwashed fruits and vegetables, unpasteurized dairy products, such as goat's milk.

There are two populations at high risk for infection with *Toxoplasma gondii*; pregnant women and immunodeficient individuals. *Toxoplasma gondii* can be transmitted directly from pregnant woman to unborn child when the mother becomes infected during pregnancy. Congenital infection is of greatest concern in humans. About one-third to one-half of human infants born to mothers who have acquired Toxoplasma during that pregnancy are infected. The vast majority of

women infected during pregnancy have no symptoms of the infection themselves. The majority of infected infants will show no symptoms of toxoplasmosis at birth, but many are likely to develop signs of infection later in life. Loss of vision, mental retardation, loss of hearing, and death in severe cases, are the symptoms of toxoplasmosis in congenitally infected children.

3. CONCLUSIONS

1- The positive diagnosis for toxoplasmosis is not easy to confirm

2- The final diagnosis in this disease is based on the combination of clinical signs, medical history of the patient, exclusion of other potential causes and positive response of the specific terapy

3- Such cases invite us to update the approach to toxoplasmosis, as clinical signs, diagnosis and treatment in cats as well as potential zoonotic treat for humans

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ANATOMIC AND CLINIC ASPECTS IN THYROIDIAN CARCINOMA IN DOGS

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Key words: thyroid cancer, folliculary carcinoma, adenocarcinoma

SUMMARY

The authors present aspects of thyroidal adenocarcinoma in dogs. This study included 4 cases form the medicine Department of the Faculty of Veterinary Medicine Bucharest. Every patient benefits of an ample physical examination and an ultrasound evaluation. In advance, it has been done a serology dosage of thyroxine T4 and triiodthyronine, T3. The final diagnosis was given by the cytological examination, the results were: thyroidian adenocarcinoma and follicular carcinoma. After the surgical intervetion on the thyroid gland, samples collected were send for the histopathologic examination.

Thyroid tumors in dogs are relatively rare, approximately 1 -2% of total tumors in dogs. Many of these small dimension thyroid tumors remain clinically undiagnosed and are identified on necropsy. It has been noticed that only 2/3 of the patients are diagnosed in the life time, by laboratory findings. The dogs with primary thyroid tumors are bring to the clinical examination because their owners observe the hypertrophy of the thyroid gland, without specific clinical signs of hypo- or hyper-secretion of the thyroid hormones. The primary thyroid tumors in dogs are usually non-secretory. The presence of a secretory thyroid tumor represents the only natural cause of hyperthyroidism in dogs identified until now. Thyroid hyper-function is extremely rare in dogs, even if a secretory thyroid adenoma exists, because it is known that this specie is strongly resistant at the thyrotoxic effect of a high concentration of T4 in the circulating blood. Some studies has been done, suggesting that are needed doses 20 times higher than the standard dose of T4 for inducing the thyrotoxicity in dogs. In the situation in which the thyroid gland is 75% destroyed by a growing thyroid tumor, the clinical signs of hypothyroidism could appear.

1. MATERIAL AND METHOD

The study has been done on 4 cases of canine specie selected of those presented at the Internal Medicine Clinic Department of the

Faculty of Veterinary Medicine Bucharest and whose clinical findings oriented the diagnose to thyroid neoplasia.

For precising the diagnose, the patients followed the next protocol: anamnesis, physical examination, ultrasound examination and laboratory data: serology, T4 and T3 dosage, fine needle aspiration cytology and, after surgery, histopathologic examination. The most valuable data obtained from the investigations of these patients were those offered by the imaging findings and the results of the cytomorphologic examination, which determined the differential positive result. The ultrasound gave us supplementary data for differentiating thyroid neoplasia for thyroid cysts. The cytology was performed by direct or ultrasound guided fine needle aspiration of the modified tissue.

2. RESULTS AND DISCUTIONS

Physical examination revealed in all 4 cases a ventral cervical mass that can be palpated. All dogs had no clinical signs for a long period of time, so the owners appreciated that the tumor developed slowly. The dry cough and dyspnea appeared long time ago (between 12 and 18 month ago) and resistant to treatment, are frequent clinical signs in such disease. Two of these dogs had lower voice and partial voice loss, one of the dogs had polydipsia, polyuria and weight loss in the last 6 months and another dog had pale mucosa, tachycardia and tachypnea. In all cases it could be observed the enlargement of the retro-faringian lymph nodes, as a result of the locally dissemination of the tumor or of the lymphatic obstruction. At the physical examination of the thyroid region of these 4 dogs it could be noticed a solid tumoral mass, insensible and with unregulated aspect. The modifications were bilateral and asymmetric. The serology's values of T3 and T4 were in the normal range, which confirm that this kind of tumors are usually non-secretory. The ultrasound revealed major modifications of the dimensions of the thyroid lobes, bilateral in 3 cases and unilateral in one case. The thyroid lobes appeared unregulated, with hypo- and hyperchoic areas, which deformed the thyroid capsule. In two of these patients thyroid cysts could be seen. These are unicameral non-echoic areas into the thyroid parenchyma, with unregulated aspect. The cytomorphologic result gave the differential positive diagnostic in all 4 cases, as following: 2 thyroid adecarcinoma, one colloidal carcinoma and one follicular carcinoma highly malignant.

3. CONCLUSIONS

1- It is the first diagnostic of thyroid carcinoma put in Romania

2- The cytomorphologic types diagnosticated are: - adenocarcinoma - follicular carcinoma - colloidal carcinoma

3- The solid form (follicular type) is higher malignant than the colloidal carcinoma.

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BIOFEEDBACK IMPLICATIONS IN PRACTICAL VETERINARY HOMEOPATHY IN DOGS

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Key words: biofeedback, veterinary homeopathy, dog, emotions, enviroment

SUMMARY

This article shows the use of biofeedback tehnic in veterinary homeopathy practice. We wanted to demonstrate that using the scanning of a large variety of frequencies from the animal's body, we can identify with much more accuracy the modalities and miasms used in clasical homeopathic diagnosis. We compared and supplemented the case history acquired from the owner with the biofeedback results and decided on the best remedy for that case in that certain moment. We obtain the results faster and more precise than in a clasical clinical observation.

Through homeopathic diagnosis and treatement we want to investigate the patient in a holistic way, bringing forward the phisical symptoms along with the enviromental influence and emotional stress factors. With classical methods, the time needed for a homeopathic diagnosis is rather long, somethimes over three hours. Using the biofeedback technic, this is reduced to half an hour, witch is a more convenient amount of time. Biofeedback assures, not only a faster homeopathic diagnosis, but a more precise one, doubling the experience of the doctor. In this article we took one hundred and fifty different cases and treated them with homeopathic remedies, after a biofeedback analisis to confirm and, in some cases, suggest the best remedy. We watched the result and reanalised the cases after a two week period.

The aim of our study was to be more efficient in determining the homeopathic remedy, not only through the more precise diagnosis, but also reducing the length of the examination.

MATERIALS AND METHODS

Other than the usual methods used in homeopathy wich consist in the conversation with the owner and the inspection of the patient, we used the QXCI dispositive, the harness for the body or neck (in really big dogs), legs and the pads for the animals with a greater sensibility,

who can't be handled. There is also the software on the laptop, used to translate the electromagnetic impulses into a more specific language.

The method that is used for the biofeedback consultation consists in scanning the body for 9000 frequencies, each associated with a different compound. It operates at biological speeds (up to 1/1000 of a second) charting the resonance or response of the body to these frequencies, comparing them to a norm and ranking them in degree of reactivity, identifying both acute and chronic imbalances.

The animal tested is connected to the biofeedback system with a body/head harness and limb straps. The device is then calibrated and the test scan proceeds for about four minutes, measuring the resonance or response of the body to the 9000 items (including minerals, vitamins, toxins, allergens, viruses, pathogens, organ functions, etc.).

RESULTS AND DISCUSSIONS

For each case we followed a certain pattern, starting with the calibration, after wich we determined how much stress affected the patient, how much tension was there in its body and how fast could it heal, regenerate after some injuries. Based on the testing, the response of the body showed us the most important reactions of the animal to stress and enviroment, wich we can use in homeopathy, because we cannot ask the patient how it reacts in cold/warm weather, in a damp/dry place, and we cannot determine very often what it feels or what emotions hunt it.

Each patient receives an analisis on the main risks affecting it's health, along with an emotional chart that shows how the most predominant feelings work to determine the reactions, fears and attitudes. This features are very important in homeopathic practice, because they feel the gaps that the lack of comunication between animals and humans create.

The informations that we receive from the biofeedback technic are a great completion for the usual case history we can find from the owner's perspective.

One of the cases was a male Cocker Spaniel, five years old. It had a bloody surgical wound without the tendency to heal. The patient had been adopted by a family for about 3 weeks and it hasn't adapt. He was constantly running from them, trying to escape the new home.

After our investigation with the biofeedback technic we recomanded a homeopathic remedy, to be administrated in the clasical way for 3 weeks. We saw this patient again 3 weeks later and his ownwers told us

not only the bleeding stoped the next day after the first administration of the remedy, but their dog changed its atitude towards them, becoming more docile and loving.

The remedy we chose after the investigation with regular homeopathic methods and the biofeedback technic turned out to be a constitutional one, witch is the best choice in homeopathy, because it adresses, not just the local and momentary disturbance, but the entire balance of the patient's body and mind.

Another case was a three years old teckel bitch, who presented itself with a repeated cough. It's owner explained that he had more dogs at home, all with the same symptoms. They used to cough when they were agitated, when the ownwers came home or left for work, but never when they played or ran one after the other.

After the complete inspection (homeopathic and with the biofeedback technic) we chose a remedy. The dog whom we examined recovered the next day, the others in the next few days. The symptoms were stopped and the cough did not get chronically.

Another bitch, a puppy this time, cocker spaniel, presented itself with diarrhoea, queasiness and a perticular behaviour. After it throws up, it run and hides. It shakes a lot at temperatures under 20°C. From the classical homeopathic experience we had to choose between two different remedies. We used the biofeedback technology to find out which one is the right one for the case in that precise moment. After just one day of administrating the correct dose, the dog got better.

A two years old dog, labrador retriever, came o a hip dysplasia and a numb left hind leg. It was very gentle, loved to eat, obedient, prefers the fresh air and hides from the sun. The excesive obediance was the key trait, and with a confirmation of 97% compatibility from the biofeedback system, we give it the adequate remedy. After 5 months, its hip was well and the character stronger.

A five years old chow-chow male had diarrhoea and skin problems. Every time his owners left for the holidays or for work, it began scratching until it got to the flesh. When they were at home, the problem faded. It hated the cold, the wet weather and didn't like stangers.

We chose a remedy with the help of the biofeedbak system and in two weeks it didn't have any scratcing fit. We recomended this remedy every time when they left it alone for a longer period of time.

CONCLUSIONS

1. Using the biofeedback system allowed a faster identification of the homeopathic remedies and dillutions at the moment of the analisis.

2. The colaboration between the classical homeopathy and the biofeedback investigation permited a more precise identification of the remedies useful to that moment in time.

3. Using the biofeedback technic is easy to verify the progress of the patient after the homeopathic treatement, because it shows all the organic, enviromental and emotional changes that took place from the last visit, to check up the efficiency of the chosen remedy.

4. The biofeedback technic doesn't choose only the right homeopathic remedy, but offers a complete scanning of the homotoxical status of the body.

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ASSESMENT OF IFN Γ SECRETION IN DOGS WITH TYPE I DIABETES USING ELISPOT ASSAY

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Key words: IFNγ, ELIspot

SUMMARY

Investigations were performed on healthy dogs and type I diabetes dogs, using a quantitative immunoassay ELISPOT for determination of single/cells secreting a cytokine (IFN gamma). It is suppose that type I diabetes on dogs is an immune disease where NK cells and T lymphocytes can lyse pancreatic islet cells.

Interferon γ is an essential cytokine for innate and adaptative immunity, secreted mainly by T lymphocytes, NK cells and dendritic cells. Secretion of IFNγ by NK cells has significant immunological effects as enhanced proliferation of T helper lymphocytes and modulation of immune response, activation of antigen presenting cells, stimulation of xenospecific response of cytotoxic T lymphocytes, sensitizing of T lymphocytes to the effect of IL2.

T lymphocytes – cells with cytotoxic activity, are involved in cellular and humoral immune response by IFNγ secretion, through recognition of antigens presented by CMH I and II restriction and inhibition of other cells. Dendritic cells are antigen presenting cells by association with CMH class II molecules and able to stimulate T lymphocytes. They have a lower capacity of cytokine secretion, only small quantities involved in initiation and stimulation of primary immune response.

Therefore, ELISPOT assay was used for a functionally asessment (IFNγ) of these cells involved in the generation of immune response of the organism.

MATERIALS AND METHODS

1.1. Biological materials were represented by whole blood samples with anticoagulant (K3-EDTA) from clinically healthy dogs (n = 5) and dogs with type 1 diabetes (n = 32).

1.2. Separation of peripheral blood mononuclear cells (PBMC) from dogs was performed by centrifugation in density gradient, using Percoll as medium for separation and keeping cell viability.

1.3. Quantification of IFNγ secretion was performed by ELISPOT assay (ELIspot Canine IFNγ, R&D Systems.Inc, USA), following "in vitro" cultivation and stimulation with 5 μg phytohemagglutinin / 1 ml RPMI 1640 medium.

This technique has some outstanding parameters (sensibility of 1/100.000) use two high – affinity antibodies, directed against different epitopes of the same molecule, one of these (capture antibody) is pre-coated onto a PVDF membrane of the microplate and the other (detection antibody) is labeled with biotin. The stimulated cells are cultivated in the microplate wells and the secreted cytokines will be captured by the coating antibody.

Following a washing step and removing of cells, the captured cytokines will be bounded by the detecting antibodies and streptavidin – AP conjugate. The chromogen – substrate solution will give a colour reaction (blue – black colored spot) at the sites where cytokines was bounded.

The test requires an initial step for separation of mononuclear cells by centrifugation in Percoll gradient, density of 1,077 g/ml, at 400 *g*, 30 minutes at the 20^0C followed by cultivation of separated cells in RPMI 1640 medium with 10% foetal bovine serum, at 37^0C in atmosphere with 5% CO_2 for a period of time (18 hours), after a stimulation with 5 μg phytohemagglutinin / 1 ml RPMI 1640 medium.

The last step is the immunoassay detection of secreted cytokines. The controls are: **positive control** - recombinant IFNγ, reconstituted with 250 μl of RMPI 1640 medium; **negative control** – unstimulated cells, in equal amount with those stimulated; **antigen control** – medium for cell cultures; **detection antibody control** – phosphate buffered saline.

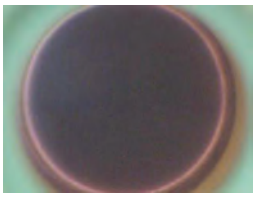
The counting of cells was performed using a Bürker-Turk hemocytometer. The counting error was about 5 – 8%. The samples were tested in two wells with $2,5 \times 10^4$ cells/well.

2. RESULTS AND DISCUSSIONS

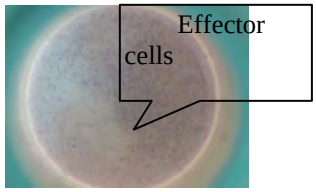
2.1. The qualitative asessment

The qualitative asessment was performed using CETI stereomicroscope and the digital camera and soft Image-Pro EXPRESS for the pictures.

The representative aspects for canine ELISPOT IFNγ asessment are presented below.



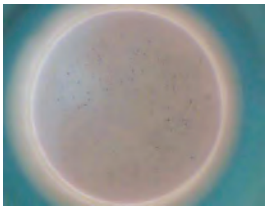
Positive control – recombinant IFNγ



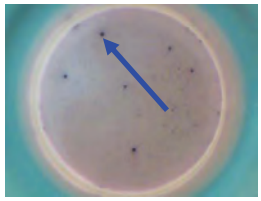
Negative control – unstimulated cells

RPMI 1640 – background control

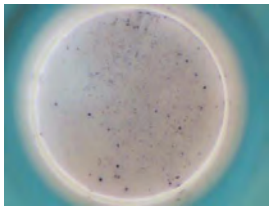
PBS – detection antibody control



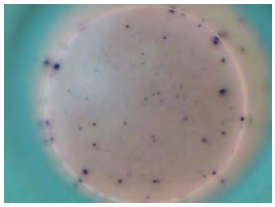
Low secretion of IFNγ, small spots



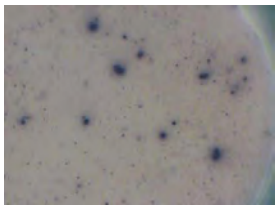
Low secretion of IFNγ - small spots (arrow).



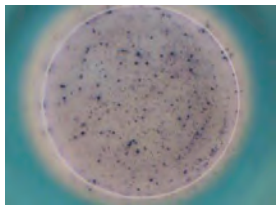
Moderate secretion of IFNγ - small spots



Moderate secretion of IFNγ - small and big spots



Moderate secretion of IFNγ - detail.



Strong secretion of IFNγ.

Table no. 1

The qualitative assessment of results obtained by ELISpot immunoassay using ELISpot Canine IFNγ kit, from canine MNC in vitro *stimulated with phytohaemagglutinin - 5 μg / 1 ml RPMI 1640 medium*

Nr. crt.	The studied disease and the number of cases	Qualitative asessment			
		Spot numbers	Response at phytoheamgglutinin stimulation	Size of the spots and the ratio among them	Intensity of spot colours
1	Clinically healthy dogs n = 5	Medium number	Increased	Mixt	Homogeneous secretion of IFNγ
4	Diabetes n = 32	Medium number	Moderate	Majority small sometimes medium, no large spots	Diffuse colour, moderate secretion of IFNγ

2.2. Semiquantitative asessment

Table 2

The semi-quantitative assessment of results obtained by ELISpot immunoassay using ELISpot Canine IFNγ kit, from canine MNC* in vitro *stimulated with phytohaemagglutinin - 5 μg / 1 ml RPMI 1640 medium

Nr. crt.	The studied disease and the number of cases	Semiquantitative asessment of spot numbers*	Areactive spots	The total numbers of identified effectors cells	
				n	%
1	Clinically healthy dogs n = 5	287 ± 35	71.75 ± 8.75	358.75 ± 43.75	1,435 ± 0,175
4	Diabetes n = 32	162,2 ± 1,5	81.1 ± 0.75	243.3 ± 2.25	0,973 ± 0,009

* from $2.5x10^4$ effector cells / well

The following formula was used for the qualitative asessment of the results: from the number of spots counted from each well were subtracted the number of spots from the negative control (unstimulated cells) resulting the real number of spots. The unstimulated effector cells

from the negative control are represented by spontaneously producing IFNγ cells.

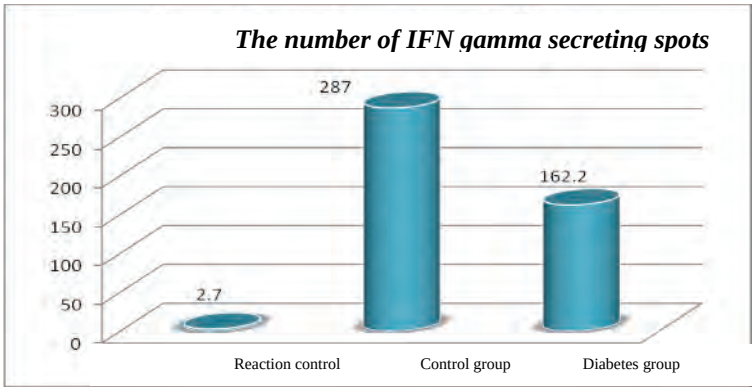


Fig. no. 1

The semi-quantitative assessment of IFNγ secreting spots from canine MNC* in vitro *stimulated with phytohaemagglutinin - 5 μg / 1 ml RPMI 1640 medium (ELISpot Canine IFNγ)

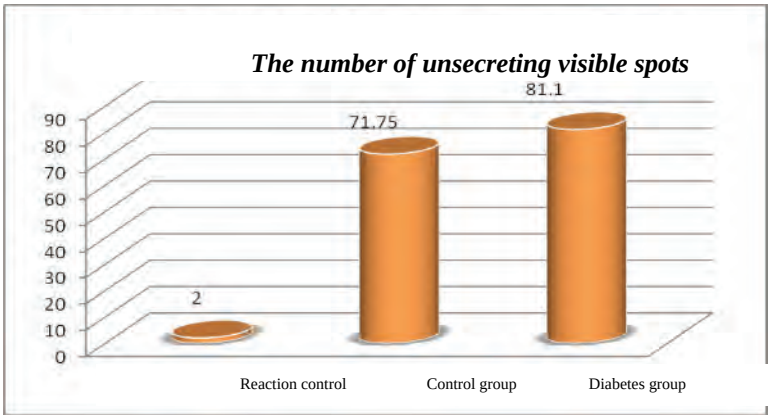


Fig no. 2

The semi-quantitative assessment of IFNγ unsecreting spots from canine MNC* in vitro *stimulated with phytohaemagglutinin - 5 μg / 1 ml RPMI 1640 medium (ELISpot Canine IFNγ)

3. CONCLUSIONS

3.1. The immunoassays ELISPOT have an outstanding sensibility and allow the functional asessment of cytokine secreting cells, with a broad feasibility in immunology research in different areas (prediction of infectious risk in transplantation, vaccine development, autoimmune

diseases, cancer research, allergy, viral infection monitoring and treatment).

3.2. It was found a large number of areactive spots in type 1 diabetes; the reactive spots have small size, low colour and diffuse – it can be interpreted as a low secretion of IFNγ.

3.3. The reactive spots are approximately 50%, small size and diffuse colored, which means a moderate secretion of IFNγ and a delayed reaction at the mitogen stimulation.

3.4 These moderate decrease of IFNγ secretion could suggest o possible corellation with the distructive effect in the pancreas against the β cells by a mobilisation of peripheric NK cells at this level.

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STRUCTURAL ASPECTS CONCERNING THE LIVER IN *GALLUS DOMESTICUS*

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Key words: broiler chicken, liver, histology.

SUMMARY

The *Gallus domesticus* species is permanently regarded to as a reference point in the structural approach of the internal organs in other galinaceeas and more. From this point, the importance of researching the macro- and microscopical elements is seen. The study presented here points out a series of anatomical aspects, especially the histostructure of the liver. The particularities of the two sides of the liver, the ventral and the visceral sides, are described, followed by the highlighting of the organization of the hepatic parenchyma in longitudinal and cross sections, which will later on facilitate the interpretation of the data concerning the pathology of the liver.

The liver (*hepar*) appears to be located in the median area of the body, behind the heart, and it is composed of two lobes. The left lobe (*lobus hepaticus sinister*) has a polyhedral aspect, is much smaller than the right one, and occupies the area between the third thoracic vertebra and the fourth lumbosacral vertebra. Its caudal side is divided into a caudo-ventral side and another caudodorsal side, by a fissure running through the cranial margin. The right lobe (*lobus hepaticus dexter*) is globular and it extends between the third thoracic vertebra and the fifth lumbosacral vertebra. Dorsally, the connection between the two lobes is contiguous to the esophagus, the gizzard, the spleen, the jejunum and the duodenum. In adult birds, it comes in contiguity even with the right testis or the ovary. The cranial region of the gizzard touches the visceral side of the liver. The caudal vena cava, which crosses the cranial region of the right lobe, reaches the dorsal limit [4,7,8].

In birds, the basic structure of the liver resembles the one met in mammals, without having an obvious pattern. It consists of a reduced capsule and of the hepatic parenchyma.

The capsule is formed of a dense connective tissue that penetrates the liver with branches from the hepatic artery and from the portal vein, forming delicate septa that incompletely separate the parenchyma into lobules. The capsule is doubled by the peritoneal serosa on the exterior.

The hepatic parenchyma is represented by hepatocytes. They are shaped similar to a pyramid, and they usually group into four or six

cells, around a bile canaliculus, as it is shown in cross sections. The hepatic cells have cubic aspect and they are arranged two by two, constituting rows as another aspect in longitudinal sections can be observed. Between these rows, between the cells, there are bile canaliculi with no proper wall, but with their lumen described only by the apical membranes of the near hepatocytes. To the periphery of the lobule, they are continued by very short ducts, canals of Herring, whose wall is lined by simple squamous epithelium. Due to those, the connection with the biliary ducts situated in the portal space Kiernan is established [1,5].

The rows of hepatocytes are separated from the sinusoidal capillaries by the space of Disse, constituting a radial network that starts from the centrolobular vein and to the periphery of the lobule, establishing a vast portal system between two veins: the centrolobular vein and the interlobular vein. The endothelium of the wall of the sinusoidal capillaries is formed from endothelial cells, rare Kuppfer cells and basal membrane, while reticulin fibers and mesenchymal cells structure the space of Disse [2,6].

The biliary ducts (*ductuli biliferi*) and the blood vessels pervade into the liver by the hepatic hilum that can be seen on the visceral side. Also, the right lobe presents the gallbladder (*vesica fellea*), which is fusiform. The bile from each hepatic lobe is drained with the help of a hepatic duct. Therefore, there will be a hepatocystic duct (*ductus hepatocysticus*), that will guide the bile from the right lobe to the gallbladder, a cystic duct, that will open in the distal portion of the ascendant segment of the duodenum, and a hepatic duct, that will start from the left lobe and end in the ascendant duodenal segment, too.

The structure of the gallbladder is formed of mucosa, muscularis externa and serosa.

The very pleated mucosa presents a simple columnar epithelium of medium height, accompanied by *lamina propria* that is rich in elastic fibers and thick. The muscular coat has an inconstant arrangement of the layers, the internal longitudinally layer alternating with a circular one and an external oblique one.

The biliary ducts have simple cuboidal epithelium, reduced corion and some smooth muscular fibers.

The blood vessels of the liver are represented by the left and right hepatic arteries (*a. hepatica sinistra, dextra*) and by the hepatic left and right portal veins.

The nerves found in this region form nerve plexi that are directed by the hepatic arteries [1,3,9].

1. MATERIALS AND METHODS

For the morpho-topographic and histostructural study, 16 birds that belong to the *G*alliformes order, of different genres, and aged between 1 day and 10 weeks old, have been used.

The macroscopical researches were done on fresh cadavers after the slaughtering of the birds by sectioning their femoral artery or by sectioning their jugular veins and the inferior cervical arteries. In order to assure a proper blood emission, the slaughtering of the birds was preceded by tranquilization with intramuscular Ketamine, 10-30 mg/kg, the dose rigurously adapted to their weight. Regarding to the method of morpho-topographic study, regional stratigraphic dissection was used, followed by the photographing of the most important aspects and by measuring the interested parts after detaching.

For the optical microscopy researches, the interested pieces have been histologically processed this way obtaining seriated cross or longitudinal sections. These sections were stained with Hemalaun-Eosin, PAS and Silver impregnation methods. They were examined with Nikon-Labophot 2 optical microscope and photographed.

2. RESULTS AND DISCUSSIONS

Aborally, the liver limits the heart. The left lobe is smaller than the right one. The ventral margin presents a profound cleft in the surface, which separates the territory into a left lateral side and a right medial side. This cleft deepens as the birds age. Aborally, the ventral margin reaches the gizzard.

The right lobe is well developed. The contour accentuates the globular aspect. The margins of this lobe are mostly continuous, excepting the middle region of the dorsal margin, where an indentation is formed. The impression made by the heart in the dorsal margin can be seen on the territory of both lobes.

The visceral side is concave and uneven because of the numerous prints left by the esophagus, the glandular stomach, the gizzard, the spleen, the jejunum and the duodenum, the most obvious and deep print of all being the one made by the proventriculum. On this side, there is a depression in which the hilum of the liver and the elongated gallbladder can be seen.

Histostructurally, the liver is characterized by a particular arrangement of the hepatocytes, organized with the help of a network of

delicate conjunctive reticular fibers. Also, the thin capsule is not capable to sent septa so that it individualizes the hepatic parenchyma into well contoured lobules. For these reasons, the whole hepatic territory will present itself in two possible variants, depending to the way the sections were made. Thus, in cross sections, the hepatocytes group by four to six around a bile canaliculus. This aspect is very resembling to the constituting of the serous acini in the exocrine pancreas and it gives the cells a pyramidal contour. The groups of hepatic cells can compose short columns or they can show themselves either isolated, either agglomerated, depending on the path of the sinusoids and their dilatation state (Figure 1).

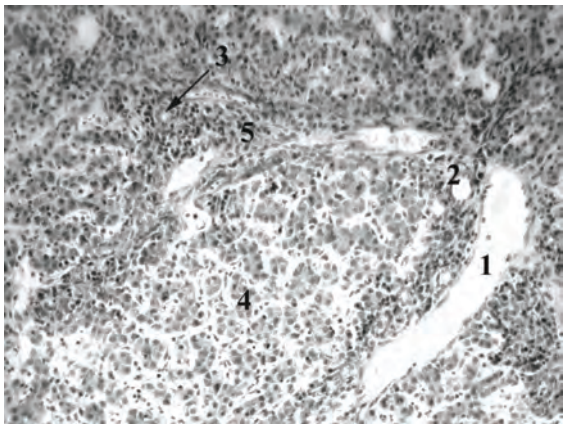


Figure 1. Liver parenchyma aspect, in transverse section/ HE, ob.10x
1. Hepatic portal vein branch;
2. Hepatic artery;
3. Biliary duct;
4. Hepatocytes;
5. Kiernan space.

The longitudinal sections made show the polygonal shape of the hepaticytes, which will be seen two by two in rows that start from the centrolobular vein to the are orientated periphery of the lobule. The lobules are inconstant in sizes, and even with the help of special methods of staining their areal cannot be exactly established (Figure 2).

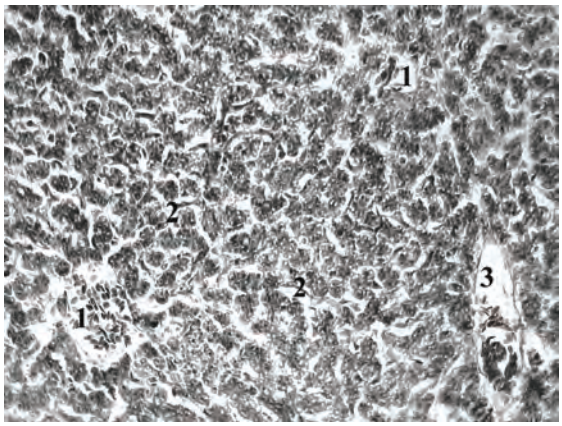
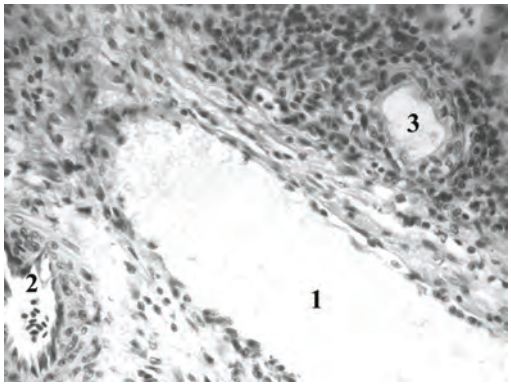


Figure 2. Liver parenchyma aspect, in longitudinal section/ Giemsa, ob.10x
1. Central vein;
2. Sinusoids;
3. Kiernan space.

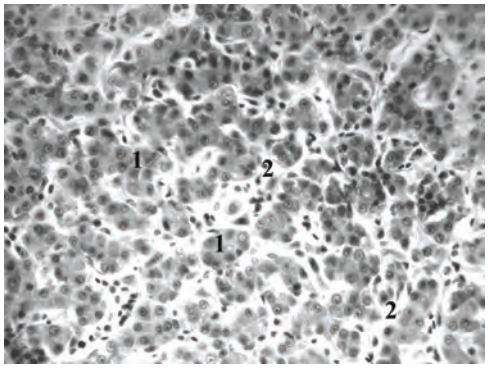
In the birds that were studied, the structure of the biliary ducts is also hard to notice in the portal space of Kiernan, where branches of the portal vein and of the hepatic arteries appear (Figure 3).

Figure 3. Detail which involve the liver parenchyma, in the Kiernan space region/HE, ob.20x
1. Hepatic portal vein branch;
2. Hepatic artery; 3. Biliary duct.



The wall of the sinusoids contains an endothelium made of slightly distanced squamous cells, rare Kupffer cells and the discontinuous basal membrane evidentiated with silver impregnation. Their diameter varies between an acceptable range, on the whole portal system which it forms, from the large, circular centrolobular vein that has an extremely thin wall and the interlobular vein (Figure 4).

Figure 4. Detail concerning the pattern of hepatocytes/ HE, ob.20x
1. Hepatocytes;
2. Sinusoids.



3. CONCLUSIONS

3.1. The ventral margin of the left lobe presents a shallow cleft in the than the one in older birds.

3.2. There is no obvious individualization of the lobules in the hepatic parenchyma.

3.3. The pattern of the hepatocytes gives a reticular aspect to the hepatic parenchyma.

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THE STUDY OF THE INFLUENCE OF SELENIUM AND VITAMIN E IN THE APPEARANCE OF SOME MORBID ENTITIES AMONG CHICKENS BRED IN A SEMI – INTENSIVE SYSTEM

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Key words: exudative diathesis, nutritional miodistrophy, encephalomacy, tor.

SUMMARY

The research on the role of selenium and vitamin E in the occurrence of some morbid entities as the exudative diathesis, nutritional miodistrophy and encephalomacy were resumed by many researchers over the time, who reached the conclusion that selenium is active in exudative diathesis, only partially active in miodistrophy and ineffective in preventing encephalomacy. (Sevastre *et al*, 2005; Mihai, 2000; Sanders, 1986)
The conclusions were based only on the prophylactic and curative efficiency of these selenium sera towards these morbid entities.
Our research was based on the reproduction of these morbid entities by giving rations that are deficient in selenium and, at the same time, it was followed to what extent the combination between selenium and vitamin E intencifies the prophylactic and curative action when talking about these morbid entities.
To feed the poultry it was used some fodder ration based on tor, but there could not miss the corn flour which raised the selenium value of the ration. (Pârvu *et al*, 2003; Pârvu, 1992)
This determined us to add sodium sulfate and iron chloride in the basic ration, which acted as interferential and stressful factors for selenium and vitamin E. (Sklan and Donoghue, 1992)
When reaching 3 to 4 weeks of administration of the fodder ration, morbidity in exudative diathesis reached 45%, while for nutritional miodistrophy got to 12.5%.
It should be noticed that the relatively high morbidity percentages occurr to young ages (1 to 2 days old), while for the 16 - 18 day old chickens morbidity is quite low; there was not registred any case of encephalomacy either.
To conclude, one can say that selenium is directly involved in the occurrence of exudative diathesis, its role in miodistrophy is questionable, and the fact that we could not reproduce encephalomacy, thisallows us to draw the conclusion that selenium has no role in its occurrence.

The current work has got the purpose to demonstrate the importance of selenium and vitamin E in the importance of appearance of some morbid entities at chickens that were bred in a semi – intensive system and which were fed fodder rations that have deficiencies in selenium and vitamin E.

MATERIAL AND METHOD

The research on the effectiveness of selenium upon some morbid entities, previously considered clinical manifestations of deficiencies in vitamin E, has concluded that selenium is active in exudative diathesis, only partially active in miodistrophy and ineffective in encephalomacy.

The problem of the relationship between selenium and encephalomacy has been resumed by some researchers. The conclusions reached by these authors are based only on the preventive and curative effectiveness of selenium salts upon these morbid entities and they are sometimes contradictory, as well as the question whether it is necessary or not to supplement poultry rations with selenium salts (Sklan D, Donoghue S., 1992).

The aim of our research was to find other material arguments that advocate for and against selenium relations with other morbid entities, and that is why we have proposed to track the following:

- To try to reproduce these morbid entities by administrating rations that are deficient in selenium;
- To determine the content of selenium in natural and experimental occurrence conditions of such morbid entities;
- To keep track of the preventive and curative efficiency of selenium, to determine to what extent the combination of selenium with vitamin E enhances the prophylactic and curative action against these morbid entities;
- We found out that solving the aimed problems, this will allow us to determine more precisely the relationship of selenium in exudative diathesis, miodistrophy and encephalomalacy, so that in the end, taking account of previous research on the selenium content of fodder, we could draw a conclusion on the usefulness of selenium, the need to introduce it or not in the poultry feeding ration, in the conditions of our country.

Our research will try to determine whether selenium plays a role in these morbid entities' etiopathogenesis or not, but without approaching the complexity of etiopathogenetic factors which are incriminated in these entities, unless their interrelationship with selenium (Sevastre *et al*, 2005).

EXPERIMENTAL TESTS TO REPRODUCE EXUDATIVE DIATHESIS, MIODISTROPHY AND ENCEPHALOMALACY

We have decided to reproduce exudative diathesis, miodistrophy and encephalomalacy using a basic tor ration, whose content was the following:

Table 1

Fodder ration with tor.

Maize	47,5 %
Torula	43,0 %
Animal fat	5 %
Calcium carbonate	4 %
Sodium chloride	0,5 %

100 g of the mixture contains:

Raw protein	25,35 %
Productive energy	181Kcal
Calcium	1,86 g
Phosphorus	0,86 g
Sodium	0,34 g
Manganese	0,79 mg
Copper	0,76 mg
Arginine	5,3 %
Cystine	0,9 %
Histidine	2,5 %
Isoleucine	5,3 %
Phenylalanine	4,3 %
Leucine	8,4 %
Lysine	6,0 %
Methionine	1,7 %
Thereonine	4,0 %
Tryptophan	1,2 %
Valine	5,9 %

This ration was composed based on tor knowing that exudative diathesis may be reproduced by using such rations. Our ration differs from those described in the literature as we did not supplemented the arginine, methionine and cystine ration, these factors being poor in our ration.

We did it because we did not have these substances, so we had to make up a ration that has got a lower selenium content in order to appreciate its role in triggering this morbid entity.

It is but known that tor is poor in vitamin E and biologically available selenium (Pârvu *et al*, 2003; Pârvu, 1992).

Moreover, as it emerged from our research, it has a content under 0.1 ppm selenium also mentioning the fact that its accessibility is reduced.

Our research was carried out on Rock and Cornish chicken breed grouped in 5 lots of 40 chicks each.

There were used 1-2 day old chicks in lots 1, 2 and 3 and 16-18 day old ones in lots 4 and 5, taking into account that exudative diathesis is more common with younger ages and at encephalomacy with older ages; miodistrophy can occur in both situations, thus we have tried to create conditions in terms of age as close as possible to those of the production of spontaneous disease (Mihai, 2000).

The chicks were fed torula – based rations created by us, for 3 - 4 weeks for the first 3 batches and for 5 weeks for the last batch.

The results obtained with our experimental reproduction of exudative diathesis, miodistrophy and encephalomacy are illustrated in Table. 2.

Table 2

Tests of experimental reproduction of exudative diathesis, miodistrophy and encephalomalacy

No.	No. chickens	Experi-mental batches	Age	Basic ration + supli-ment sodium selenite	Length of adiministration	Morbidity					
						Encepha-lomalacy		Mio-distrophy		Exudative Diathesis	
						No.	%	No.	%	No.	%
1	40	Batch 1 control	1-2 days	Basic torula ration	3-4 weeks	-	-	-	-	3	7,5
2	40	Batch 2	1-2 days	Basic torula ration + sodium sulphate	3-4 weeks	-	-	1	2,5	9	22,5
3	40	Batch 3	1-2 days	Basic torula ration + sodium sulphate and iron chloride	3-4 weeks	-	-	2	5	18	45
4	40	Batch 4	16-18 days	Basic torula ration + sodium sulphate	5 weeks	-	-	1	2,5	-	-
5	40	Batch 5	16-18 days	Basic torula ration + sodium sulphate and iron chloride	5 weeks	-	-	5	12,5	-	-

RESULTS AND DISCUSSION

The new data show that exudative diathesis occurred after 2 - 3 weeks from the initiation of the diet scheme based on tor for the first batch, having a rate of 7.5%.

This low mortality rate occurs because we did not manage to achieve a ration with a lower selenium content due to the introduction of maize flour, which raised its value.

It is interesting to notice that although the ration was somehow deficient in amino acids sulfur (methionine, cystine), there was no case of miodistrofie.

The relatively low selenium content determined us to supplement the basic ration with sodium sulfate and iron chloride to interfere vitamin E and selenium (Pârvu *et al*, 2003; Sklan and Donoghue, 1992).

The ration administration of sodium sulfate resulted in an increase of morbidity from 7 - 25.5% and concomitant supplementation with sodium sulfate and iron chloride ration caused the increase of morbidity with exudative diathesis even more, up to 45%.

Regarding miodistrophy, it is to be noted that it appeared once supplements of sulphate were introduced in the diet, but in a rather low percentage of 2.5%, percentage that grew slightly if the ration is simultaneously supplemented with iron chloride up to 5%.

Our observations highlight the fact that various morbid entities that we tried to reproduce, depend on the age the chickens are when they are introduced in the experiment.

If they are placed at an older age, meaning 16-18 days, supplementing rations with sodium sulfate and iron chloride do not cause exudative diathesis, but only miodistrophy, which in the case of simultaneous administration of sodium sulfate and Iron chloride can attain a higher percentage than with younger age, recording a morbidity of 12.5%.

The emergence in the first 2 weeks of the exudative diathesis is linked to the fact that in this period we recorded the lowest levels of selenium in tissues and therefore, the chickens are more easily susceptible to selenium deficiency.

Based on the obtained results we can say that exudative diathesis may occur as a result of poor diet in selenium, but there should not be neglected the role of vitamin E as well.

It is interesting to notice that exudative diathesis could not be reproduced anymore when our ration was introduced in the diet of 16-18

day chickens, although the ration was supplemented with iron chloride, which interferes vitamin E.

We believe that in this case, the exudative diathesis is not installed because the ration of chicks up to the age of 16-18 days has got the necessary of vitamin E especially that of selenium, which is relatively slowly eliminated from the body and it is not influenced by the stress produced by iron chloride.

3. CONCLUSIONS

3.1. It is noticed the importance of selenium in the appearance of exudative diathesis, but it should not also be neglected the role of vitamin E and synergistic action between vitamin E and selenium.

3.2. Regarding miodistrophy, this appears to be related more to the presence of vitamin E and sulfur amino acid content, since it occurred only when stressing factors were used, although there was a predisposing background of the ration, namely the slight deficiency in selenium and sulfur amino acids.

3.3. Miodistrophy is less related to the selenium deficiency as it occurs in a higher percentage at an older age, when the need of selenium was somehow assured by the normal diet administered up to the age of 16-18 days.

3.4. The very low percentage of morbidity makes us believe that both vitamin E and selenium have a limited role in etiopathogeny of miodistrophy, being more the result of deficiency in sulfur amino acids, but this does not mean that we should completely neglect the role of selenium and vitamin E.

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CASE STUDY – ENCEPHALITIS IN A 4 YEAR OLD HUSKY

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Key words: dog, encephalitis, neurology

SUMMARY

In this paper we are discussing the case of a 4 year old female Husky dog, which first arrived in our clinic with fever and neurological symptoms. After taking the case history, the clinical, neurological and complementary exams, the diagnosis given was that of encephalitis and the appropriate treatment was started (the treatment was adapted from human medicine).

A dog was brought to our clinic that had been experiencing fever and neurological symptoms for a week. This patient had been treated previously in a local clinic in Piteşti, but the symptoms had gotten worse. The clinical signs improved only after 3 weeks of treatment, and the patient was considered clinically healthy 3 months after the symptoms had first appeared. The treatment was started immediately after the diagnosis was confirmed through a MRI exam and was continued for three months.

MATERIALS AND METHODS

The case was studied and treated at the Medical Clinic of the FMV, Bucharest; the clinical and neurological exams done in the Medical Clinics and for a certain diagnosis a MRI was done with the help of our colleagues from Colentina Hospital.

For this case, the steps in diagnosis and treatment were as following:

- Case history
- Clinical exam
- Blood exam and biochemistry exam
- Serum exam (Carré disease, babesiosis)
- Eye exam
- Radiologic exam
- Neurologic exam

RESULTS AND DISCUSSIONS

In October 2008 we received the case of a 4 year old Husky female dog with uncertain symptoms but that had been having fever for a week (40,2°C – 40,9°C). The dog had been treated in a local clinic in Pitești using antibiotics and fever relieving drugs, but the fever persisted and the clinical signs worsened.

After a detailed history was taken the clinical examination revealed the following:

- Apathy, depression
- Ataxia, astasia, posterior limbs dismetry;
- The animal was present minded but preferred the lying position;
- During walking the animal held its head stiff and felt pain when moving its head;
- The proprioception is delayed on all four limbs, more on the posterior two;
- The spinal reflexes were normal;
- The cranial reflexes seemed normal but at the eye examination horizontal nistagmus was detected in the right eye while in the left eye we detected optical papilla edema;
- The cutaneous and the perineal reflexes were normal.

After the clinical examination we decided to undergo the following:

- Hematology exam:
  - Significant neutrophylia
- Biochemistry exam:
  - No detectable changes
- Urine analysis:
  - Normal values
- Carré disease test:
  - Negative
- Babesiosis test:
  - Negative
- Radiologic exam:
  - Small developing osteophytes detected on the lumbar spine

The treatment underwent was as such:
Cefort (500mg x 2 times/day i.v.),

Ancesol (2 ml/day i.m.)
B1 (1 vial/day s.c.)
B6(1 vial/day s.c.)
Piracetam (2,5ml a day i.m.)
Furosemid (1 vial/day i.m.)

We presumed that the dog was suffering of encephalitis or viral meningoencephalitis and thus we asked for a MRI exam to determine the definitive diagnosis. The diagnosis after the MRI was that of encephalitis in the cerebral hemispheres.

After three days of treatment the temperature remained at a value of 40^{o}C, and the dog's general status declined to that of the dog remaining in permanent laying position. We changed the antibiotic to Doxicicline (10mg/kg/day), in one dose, for 10 days. To relieve the intracranial pressure we recommended Manitol in slow per (100ml/day i.v.) and Dexametasone (40mg/48 hours s.c.).

After this treatment the patient's status improved, regained her appetite, started to get up on her feet but moved only supporting herself on different objects.

The treatment continued with Piracetam and B vitamins, but unfortunately the neurological symptoms reappeared after just a week, more aggressive (posterior limbs instability, crossing of the legs when walking, tendency to walk in circles – both ways). Because of this we started treatment with antibiotics that can pass the hemo-encephalic barrier: Bancomicine (250mg twice a day in slow perfusion) and Tienam (250mg twice a day in slow perfusion), for 10 days. This treatment was supplemented with Oxibral (1cps/day, for 30 days) after we initially tried using Cerebrolizin (2,5ml/day, i.m.) but this drug gave the dog a high excitation state. For the vestibular syndrome we used 12mg Betaserc (1 tablet/day, for 30 days).

The patient came back for a check-up 14 days after the treatment presenting a good general condition, and the neurological symptoms were completely improved. The treatment was continued orally with:

- Omega-3 - 800mg x 2 times a day, for a year;
- Piracetam - 400mg/day, for 90 days; afterwards 400mg/day, 10 days a month, for a year;
- Betaserc – 12 mg/day, for 30 days; 10 days a month, for three months;
- Oxibral – one tablet/day, for 30 days; 10 days a month, for three months;

The patient comes for a check-up every three months, her general condition is good, the blood exams show no change for the worse and the neurological symptoms are absent.

CONCLUSIONS

1.1 When examining a case with undefined symptoms we must establish if it is indeed a neurological case or not.
1.2 The case history, the clinical exam and the neurological exam ar as important as the laboratory tests.
1.3 The differential diagnosis in this particular case was between encephalitis/meningoencephalitis, stroke (hemoragic lesions), infections/inlamations, metabolic disorders, but a sure diagnosis could only be determined through a MRI exam.
1.4 A proper treatment started in due time is important because by not doing so the lesions of the nervous system may become permanent.

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TOXIC SUBSTANCES - TRIGGERS OF EPILEPTIFORM EPISODS IN DOGS AND CATS

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Key words: toxic substances**,** antiepileptic drugs, supportive treatment, dog, cat.

SUMMARY

Epileptic manifestations appear due to an abnormal and hypersyncronous neuronal activity in the central nervous system. This type of activity is known in specialized literature as "neuronal electric storms". Recurrent seizures are one of the characteristic manifestations of any nervous system which has an individual sensibility to a specific type of stimulus. No connections were found between type of seizures and the trigger (days of the week, the phases of the moon, vacations), but there are well-known triggers which are: flashing lights, lack of sleep, starvation, big noise, toxic substances.

In the present study we took into discussion a cat and a dog with epileptiform manifestations caused by a intoxication with several toxic substances. Seizures episodes appeared several hours after the toxic substance ingestion in both cases.

In the cat, we applied a therapy with antiepileptic drugs because the seizure episodes were numerous and this therapy was supplemented with supportive treatment. In this case we managed to make an MNR examination, and it reveals an abnormal area in the frontal cerebral lobe, with edema. Cerebral edema was sustained once more by the ophthalmologic exam which revealed a retinal edema.

The dog in the study had a dermatologic affection and needed a very toxic medication. The dog was neglected after the medication and it ingested the toxic substance. Its clinical manifestations were seizures, hypersalivation, loss of behavior. The dermatologic treatment was immediately interrupted and the therapy applied consisted in antiepileptic drugs and supportive treatment.

Within several days, both the cat and the dog recovered and never had such type of problems although the antiepileptic drugs were gradually withdrawed.

The word "epilepsy" comes from the Greek word "epilepsia" that means "to be taken", "to be attached". This disease has been described in humans since antiquity. The Greek philosopher and Doctor Hippocrates (460-377 B.C.) believed since then that the cause of the convulsions is located inside the brain. Then, the epilepsy was associated with supernatural forces, because of the dramatic behavior (the convulsions).

The influence the human epileptology in veterinary medicine cannot be minimized. In the works since 1900 there were comparisons between convulsive symptoms in humans and dogs.

The epilepsy is one of the most common neurological diseases in dogs and cats. It is caused by the abnormal and hyper-synchronous

electrical activity of the central nervous system's neurons. This type of activity is known in the literature as "cerebral electrical storm" (Bagley, 2006; Berendt, 2008; Platt and Olby, 2005). This disease is characterized by recurrent convulsive crisis, the result of this abnormal electrical activity and of the excessive neuronal discharges in the brain (Constantin, 2002).

The prevalence of this disease in the canine population is estimated to be 0.5 - 5% and in the feline of about 0.5% (Tilley and Smith Jr., 2000). There seems to be more common in males.

No links have been demonstrated with season, phases of the moon, days of the week, but the lighting and sound stress, sleep deprivation etc. are well-known causes of the convulsions in susceptible individuals (Bagley, 2006).

This work aims the exposure of several cases of epileptiform events in patients who suspected intoxication with various substances (medicines, cleaning products, flies spray) and tracking the effectiveness of treatment as well as the subsequent developments.

1. MATERIALS AND METHODS

The study was conducted between October 2008 and July 2008 in the Faculty of Veterinary Medicine Bucharest, Pathology Clinic and Medical Clinic condition during doctoral studies.

The study on this subject was performed on two patients, a cat and a dog, with epileptiform events due to toxic causes. They reported the existence of the epileptiform events in their animals.

In the anamnesis was pursued finding of potential triggers of these epileptiform events, in both cases we observed the animals have been exposed not long ago to toxic compounds.

Case no. 1

On 3rd of November 2008 in the Pathology Clinic and Medical Clinic came an owner with a European cat, female, 2 years and three months old, having epileptiform events.

The first events of this kind have occurred two days ago, first three seizures per day, the next day occurring tens of seizures per day. The owners could not associate the seizures with joy, fright, hunger, thirst, effort, because the cat was not exposed to these stimuli, the cat being a flat one.

After a detailed history we found out that a few days ago the owners administered in the house antiparazitar sprays against flies and mosquitoes, and the cat was not being isolated in another room and

having direct contact with toxic substances and dead insects. Also during this time the cat has consumed the leaves of houseplants. Based on this information we suspected a toxic cause of these events.

The epileptiform seizures were partial and generalized, more frequent being the partial ones. The partial ones are described as follows: the animal has a thick meow, leaves in abdominal decubitus, occur tremors of the ears, lips and eyes, mydriasis, gaze and then running in all directions and striking objects.

The generalized seizures included thick meow, abdominal decubitus and then lateral decubitus, pedaling of all limbs, forced extension of the head on the neck, contraction of the masseter, hypersalivation, mydriasis, loss of urine and feces.

The partial seizures last 10-25 seconds and the generalized ones last 1-3 minutes.

Although the cat received intra-rectal Diazepam 5 mg vial, it still had partial and generalized seizures in 15 to 15 minutes throughout the night.

Between these seizures, either partial or generalized, the animal presents a state of prostration and altered mental status.

The cat presents interictal polyphagia but refuses to drink water.

The neurological exam performed when it came to the Clinic showed diminished motor and proprioceptive reflexes on the right side and lack of sensitivity on the tail. The revaluation in two hours showed the motor and proprioceptive reflexes were missing on right side of the body.

The abdominal ultrasound did not show any changes.

The eye examination showed fixed bilateral mydriasis and papilla edema of bilateral optical nerve as well as latero-lateral nystagmus in both eyes.

The parasitological examination showed that the cat is severe infected with *Toxoplasma gondii.* After finding this result, the suspicion has spread and in terms of parasitic cause of these events.

The blood biochemical examination showed only the increase of the GPT with 50% to the maximum allowable (80 U/l instead of maximum 50 U/l), the effect of the toxic substances action that the animal came in contact with. The hematological examination showed eosinophilia and neutrophilia.

Treatment was instituted with glucose saline and i.v. Acepromazine.

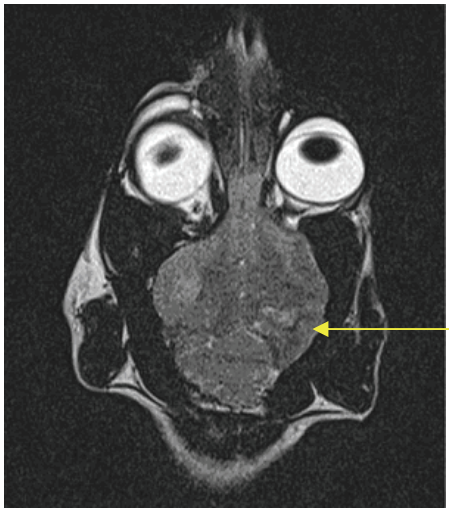
After the eye examination the treatment was completed with Mannitol, Furosemide and Dexamethasone and the animal received also

i.v. diazepam (2,5 mg) and i.m. phenobarbital (25 mg), because it still had generalized and partial epifeptiforme seizures.

The treatment against toxoplasmosis was: Daraprim (25 mg pirimethamin) 12,5 mg p.o. daily, Biseptrim (80 mg trimetoprim and 400 mg sulfametoxazol), half a tablet p.o. daily and folic acid 1 mg for the next 21 days.

Because the animal presented dramatic events, the cat owners chose to intern it in a private clinic between 5 and 9 November 2008. During this time the following treatment was given: i.m. phenobarbital 25 mg from 8 to 8 hours, i.m. Mannitol and Furosemide perfusion twice a day.

On 08.11.2008 a native cranium-cerebral MRI examination was performed that only showed on the right temporo - parietal side an area of about 2 cm with flattened appearance suggesting a cerebral edema (Fig. 1,2). All other cerebral structures did not show alteration.



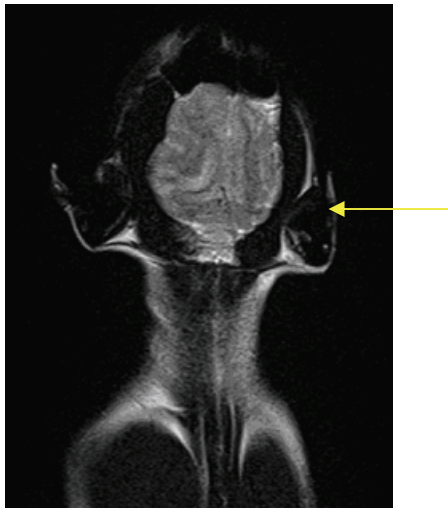


Fig.1 – Cranio-cerebral MRI, cerebral edema on the right side temporo-parietal lobe	**Fig. 2 – Cranio-cerebral MRI, cerebral edema on the right side temporo-parietal lobe**

After anesthesia performed for the MRI examination, the cat recovered very difficult, requiring 6 days of perfusion for regaining motility, voluntary urination, interest in food and water. The perfusions consisted in Ringer solution and Furosemide. To continue the cerebral edema treatment, dexamethasone (0,5 mg) was given every two days, three times .

The blood analysis performed on 11th of November consisted in biochemical analysis and blood counts. The liver injury shown by the GPT's growth remained and the blood counts showed neutrophilia, granulocytosis and eosinophilia**.**

On 15th of November the animal had a partial seizure and on 16th of November another generalized one.

The home treatment consisted in Phenobarbital 1 mg/kg twice a day and the above mentioned treatment against toxoplasmosis.

On 11th of November, 2008 at second check up, toxoplasmosis became negative and the cat did not show any epileptiform seizures. The owners did not use any toxic substances inside the house and they eliminated all the houseplants.

The eye examination performed at this date showed that the papilla edema of optical nerve was resolved.

As long term recommendations: gradual removal of the Phenobarbital.

Case no. 2

On 23.02.2009 came a Boxer Rottweiler crossbreed dog male 7 months old.

The owner told that the animal have excessive itching and hair loss.

At the general examination I saw that the animal has a generalized, pruritic and suprainfected dermatitis. It was recommended dermatological and parasitological analyses and based on these the diagnos was a parasitic dermatitis with *Sarcoptes scabies*. The dog is neither vaccinated nor dewormed. The body temperature was within the normal limits.

As treatment for the Sarcoptes scabies was recommended bathing with Amitraz solution 2 ml in one liter of warm water, two times a week for four weeks and Scabex ointment (permetrin) for 21 days.

After the first bath with amitraz solution the owners did not follow the doctor's recommendation and did not observe the animal not to lick its body, so that in a few hours an amitraz intoxication occurred manifested by anxiety, right and left manege, frequent urination and epileptiform seizures. The epileptiform seizures have the following characteristic: after anxiety, urination and manege, the dog falls inlateral decubitus, hypersalivation, rictus sardonicus, loss of urine and generalized tonico-clonic contractions. The seizure lasts about one minute and there were 3 such seizures in 12 hours. After the last seizure there also was a vomiting episode. Interictal the animal is frightened and confused and refuses to move.

Dexamethasone (2 mg), atropine (0,5 mg), hydrocortisone hemisuccinat (25 mg)and calcium gluconate (1 mg) treatment was applied. And, because the seizures did not stopped, was also given i.m. phenobarbital (50 mg).

The blood biochemical analysis were refused y the owners due topersonal reasons.

The next day the animal still has shown an impaired general condition, normal temperature and also three seizures during 24 hours. The treatment was supplemented after with perfusion twice a day with Ringer solution 250 ml, aspatofort and vitamin C. The third day the dog had only one epileptiform seizure. During all this time the amitraz treatment was stopped, and will be resumed one week after the seizures will stop and after the return of a good general condition, with the explicit recommendation to avoid the product to be ingested by the animal.

2. RESULTS AND DISCUSSIONS

Case no. 1

In this case, the toxic from the flies and mosquitoes spray caused a strong cerebral edema, shown by the eye examination performed in the first day of treatment and, consecutive, all the events that followed.

Although the cat was strongly infected with *Toxoplasma gondii*, I excluded this to be a cause of the epileptiform events, because the MRI examination showed the lack of the Toxoplasmosis cerebral cyst, the main element that could have triggered these epileptiform seizures.

The treatment was a complex one that covered, on one hand, the drainage of the toxic using i.v. perfusions and also sustaining the liver to metabolize the toxic and to eliminate it as soon as possible and on the other hand to sedate the animal in order to stop the epileptiform seizures series that could have been fatal. Because the animal had a good development and recovered due to these treatments, the anticonvulsive drug was recommended to be gradually removed, forasmuch the seizures together with all the pre, post and interictal signs were absent for a few days. This turned to be a good decision because in the absence of the toxic the animal did not have any epileptiform seizures, so it did not need anticonvulsivant drug. The only inconvenient was a dermatitis caused by the toxic contained in the drugs used against the toxoplasmosis. This was treated and completely resolved.

Case no. 2

In the case of the dog intoxicated with amitraz solution, the seizures appeared a few hours after the toxic substance ingestion and this was immediately associated with the treatment against scabies.

The treatment aimed the draining of the ingested toxic by using i.v. perfusions twice a day, sustaining the liver and the kidneys in the metabolism and elimination process of the toxic substance and ceasing the epileptiform seizures.

After the fourth day from ingesting amitraz and after the complex treatment, the dog did not have any epileptiform seizures, the general condition was improved and the further development was positive. In one week the phenobarbital treatment was eliminated. From then up to now there were no epileptiform seizures.

The treatment for scabies was followed more carefully and strictly by the owners and the parasitic dermatitis resolved completely.

3. CONCLUSIONS

3.1. In the two clinical cases we studied, epileptiform episods were caused by toxic substances intake.

3.2. The therapeutic protocol we had aimed the toxic substances removal from the organism as soon as posible, anticonvulsivant medication and hepatic supportive medication administration.

3.3. In both cases, thanks to the measures we took and to the treatment applied, epileptiform episods dissapear.

3.4. Disappearance of the epileptiform episods was possible because they represented the clinical manifestations of the toxic risk factors that did not caused irreversibile lesions in the central nervous sistem.

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HYPOGLYCEMIA – CAUSE OF EPILEPTIFORM EPISODS IN DOGS

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Key words: hypoglycemia**,** antiepileptic drugs, supportive treatment, dog.

SUMMARY

Epilepsy is a disease characterized by recurrent seizures (two or more) originating from the brain. Seizure activity arises as a sequel to abnormal hypersyncronous electrical activity of the neurons in the brain. Epilepsy is, above all, a consequence of an imbalance between excitatory and inhibitory mechanisms in the brain.
The present study's objective was to explain and to state the manifestations, treatment and the way were evolving the three patients which had epileptiform episodes consecutive to severe hypoglycemia.
We studied three dogs and we applied a hypoglycemia supportive therapy. We aimed to gain control of seizure activity by applying the right treatment. This therapy was made up of glucose, phenobarbital and hyperglycemiant substances.
One of the cases ended with death due to the severity of the illness but another case in on continuous treatment and has a good quality of health and life.
The treatment including glucagon, a hyperglycemiant hormone, borrowed from human medicine, which proved its efficiency in maintaining the glycemia at values compatible with a good function of al organs, especially the brain.
This therapy was supplemented with dexamethasone and diprophos administration, two antiinflammatory drugs with hyperglycemiant effect. The whole scheme leaded to obtaining good results in controlling the epileptiform manifestations in severe hypoglycemia in dogs.

Epilepsy is a neurological disease characterized by repeated convulsive seizures; it is a clinical status that results due to an abnormal electrical activity and due to some excessive neuronal discharges inside the brain resulted from an imbalance between neuronal excitation and inhibition (Berendt, 2008).

The stimulus that brings about an epileptiform seizure is different depending on individual. Theoretically, any brain "provoked" with the right stimulus can react with convulsions. No connection was observed between the type of the developed convulsions and suspected etiology (The Merck Veterinary Manual, 1998).

Epilepsy appears at any breed of dogs and cats. A familiar predisposition for epilepsy is met in Beagle, Golden Retriever, Labrador, Vizsla and their crossbreeds (Bagley, 2006). Epilepsy is not necessarily a condition that lasts for life, having also the capacity to auto - eliminate itself. In the human medicine, after numerous trials, there

was a period of time established namely 5 years, during which if the patient does not have any epileptiform seizures, he can consider himself free of this disease. At animals this period was established at 2 years. It is accepted that the remission of seizures to be obtained either spontaneously or by drugs induction (Platt and Olby, 2005; Tilley and Smith, 2000).

Epileptiform episods (manifestation that mimics epilepsy but is secondary to an other primary illness) appear by many reasons (toxic, infectious, vascular, renal failure, hypoglycemia, emotional).

This essay aims to track and expose the events, the treatment and the evolution of three patients that had epileptiform seizures events consecutive to severe hypoglycemia seizures.

1. MATERIALS AND METHODS

The study was conducted between November 2008 and August 2009 in the Faculty of Veterinary Medicine Bucharest, Pathology Clinic and Medical Clinic.

Was taken into account three patients that had epileptiform seizures and after detailed analysis it was found that they suffered of severe hypoglycemia.

The treatments aimed to stabilize the patients, to stop the epileptiform seizures series, to perform complete analyses to have a correct and complete evaluation of the patients and to recommend treatments, for them to have a good quality of life and an optimal state of health.

Case no. 1

On 23.02.2009 in the Pathology Clinic and Medical Clinic came a Bordeaux Dog, female, castrated, 10 years old. In the previous days the animal had some epileptiform seizures that worried the owner. From the history presented by the owner resulted that the nutrition is adequate (dry food and cow-cheese), the animal had also a few seizures in December 2008 and that the epileptiform events are generalized and partial. The generalized ones are pretty typical for an epileptic seizure, namely starts with agitation, the dog searches its owner then there comes a generalized tension, the animal falls in lateral decubitus, abundant salivation and all four limbs are rigid. The animal does not lose feces or urine, but is unconscious.

The partial seizures include preictal agitation, sterno-abdominal decubitus, and tension in the front limbs, gaze and mydriasis.

The seizures last between one and two minutes and after the seizures the animal is disoriented, have no balance in its walk and it completely recovers in about 5 – 10 minutes.

The seizures follow each other at an interval of about 10 days, but lately they have been more frequent. The dog did not have any anticonvulsive treatment.

After the veterinary examination it was found that body temperature was within normal limits, the mucous membranes are normally colored, the animal walks well, does not have any facial or muscle asymmetries, it is conscious, promptly responds to orders, there are no apparent changes to the lymph nodes.

An eye examination was recommended, but this did not reveal any change that could have been associated to the currently charged state.

The biochemical blood tests showed that the liver, kidneys and pancreas parameters are within normal limits, but the glycemia is very low, namely 50 mg/dl postprandial at 4 hours and the hematological examination did not show any changes.

The parasitological examination for babesiosis and heartworms was negative.

Because the owner has diabetes for a very long time, he measures at home the glycemia of the animal, and the return values were 32 mg/dl a jeun and 55-70 mg/dl postprandial at 2 and 4 hours. Because the animal was vaccinated up to date, the owner did not declare the presence of any toxic substances at his home and because the tests showed that the internal organs are functioning normally, we considered these hypoglycemia episodes to be responsible for the epileptiforme seizures.

The ultrasound examination performed on the animal when it came to the doctor showed the pancreas to be normal.

Some detailed analysis were recommended, including native and dye MRI (to detect a possible pituitary tumor that could have triggered there hypoglycemia episodes), but due to personal reasons, the owner refused.

As long time treatment, was recommended intra-rectal Diazepam 10 mg post seizure if the animal has more than 3 seizures in 24 hours and if the state aggravates, phenobarbital p.o. or by injection.

Case no. 2

On 26.03.2009, in the Pathology Clinic and Medical Clinic came a Boxer Dog, male, 14 years old, in status epilepticus for more than 12 hours.

The owner told that the first seizure of this type happened the previous day at 4 o'clock in the morning. During that day the animal ate grilled chicken, food that is not part of its regular menu. Its body temperature was 40°C, caused by the unstopped muscular activity.

Last night, after the seizures stopped the animal i.v. glucose (250 ml) and i.m. phenobarbital (200 mg); before the i.v. glucose perfusion, the value of the glycemia was 23 mg/dl, after that it was 33 mg/dl and after an hour it was 32 mg/dl.

In the morning of 26.03 the owner together with the animal came to the Clinics of the Faculty of Veterinary Medicine and it received 5% glucose (250 ml), calcium (1 mg) and phenobarbital perfusion.(200 mg) Due to this treatment the seizures stopped but only until 02:30 PM when a new seizure happened that repeated every 30 minutes. The seizures last 1 to 3 minutes. After 05:00 PM the animal enters again status epilepticus and it received intra-rectal diazepam 10 mg and a 200 mg phenobarbital; impressed by its dramatic seizures and without consulting a doctor, around midnight the owners gave the animal two vials of intra-rectal Diazepam (20 mg overall) and 200 mg phenobarbital. During that afternoon the animal received another 500 ml 5% glucose without measuring the glycemia.

Between midnight and 07:00 AM the second day it did not have any seizures.

The blood biochemical examination showed all the parameters being within normal limits except for the glycemia that was measured many times during the day and had very low value (20-32 mg/dl).

The hematologic examination did not show any changes but the abdominal ultrasound showed a pancreatic abnormal aspect, suspected to be an insulinoma (Fig. 1).

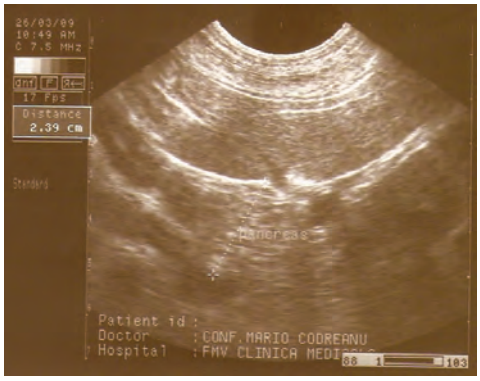

Fig. 1 – Pancreatic ultrasound apect (enlarged) in insulinoma, Boxer, male, 14 years old

After arriving to Pathology Clinic and Medical Clinic the seizures reappeared and the animal received i.m. 200 mg Phenobarbital and 250 ml 10% glucose treatment, althought the glycemia was 32 mg/dl after one hour.

Until 12 o'clock it did not have any generalized seizures and then suddenly the partial seizures reappeared located in the facial muscles (trembling lips, movement of masseter).

The salivation was very abundant. The animal's state of sedation was very profound, not being able to react to any stimulus (verbally or painfully). The traumatic tongue injuries were pronounced but the animal did not even react to repeatedly biting its tongue.

After 02:00 PM it received another 250 ml 10% glucose perfusion. The patient left the clinic sedated, the glycemia being 32 mg/dl and with the recommendation to take another 250 ml 10% glucose around 06:00 PM and Glucagen, medicine that contains the hyperglyceminat hormone, glucagon.

Case no. 3

On 26.03.2009, in the Pathology Clinic and Medical Clinic came an a German Dog and German Shepherd crossbreeds dog, male, 9 years old, that had a few days ago epileptiform seizures with loss of consciousness, lateral decubitus, pedaling of all limbs, hypersalivation, photophobia and some episodes of instability on all four limbs both in standing and walking.

The general examination showed that the body temperature was within normal limits (38.7^0C), the apparent mucosa were normal colored but the animal walk this tottery. The history showed that the animal was vaccinated and disinfected up to date, did not have contract with toxic substances indoors or outside, lives together with another two vaccinated and healthy dogs and the nutrition is adequate.

Biochemical examination and complete hematological examination were performed.

We immediately found out that the glycemia was 20 mg/dl and the animal received i.v. 300 ml 5% glucose. After an hour the glycemia was 54 mg/dl. After another hour the glycemia was 60 mg/dl. Unfortunately after another two hours the glycemia dropped again at 20 mg/dl and the tottery signs reappeared.

The animal received another 250 mg 10% glucose perfusion, this time along with diprophos (inflammatory having hyperglycemiant effect) and blood was collected to perform some special analysis,

namely the determination of glucagon and insulin in the blood. After an hour the glycemia was 30 mg/dl.

The ultrasound examination showed an enlarged left pancreatic lobe (19.3 mm). The suspicion is hyperinsulinism caused by a higher production of the enlarged lobe (Fig. 2).

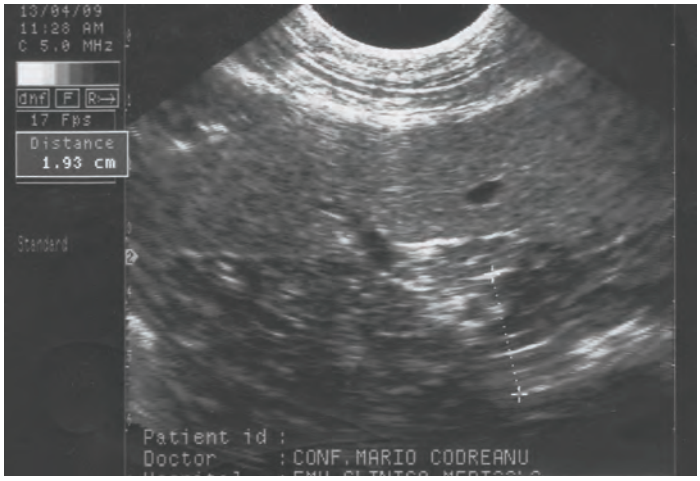

Fig. 2 – Ultrasound aspect of an enlarged pancretic lobe (1,93 cm)

The parasitological examination showed negative results (babesiosis and heartworm).

The determination of insulin in the blood had results within normal limits. The complete hematologic examination did not show any changes and the blood biochemical examination showed that all the parameters had values within the admitted limits. Because the serum calcium's value was within limits, the hypocalcemia was excluded as being a cause for these events.

The eye examination did not show any changes that is why a possible stroke was excluded too.

In the same day at 05:00 PM, the animal had a severe epileptiform seizure that lasted about 5 minutes, with all its typical symptoms.

It was recommended Glucagen (medicine that contains glucagon) twice a day. The first dose was on 13.04 at 08:00, following that for the next 5 days to receive this medicine at 08:00 AM and at 08:00 PM.

The owners were instructed to give the dog sugar and honey during the day. In the following days, glycemia was closely monitored and the obtained values are presented in table 1.

Table 1

Values of glycemia between 14.04 - 18.04.2009

Nr.	Date	Hour	Glycemia value (mg/dl)
1	14.04	14	20
2	15.04	11.30	76
		14.45	64
		21.00	56
3	16.04	7.00	41
		9.20	96
		20.30	56
4	17.04	10.50	54
		20.20	47
5	18.04	10.45	36
		12.25	93
		19.00	51

Between 21.04 and 02.05 the glycemia was between 65 and 140 mg/dl and on 02.05 had a light epileptiform seizure preceded and followed by loss of balance. Immediately after the seizure the glycemia was 29 mg/dl (11:15 AM). The animal received honey and after an hour the glycemia increased at 49 mg/dl and after two hours it was 59 mg/dl.

During the same day the animal also received dexamethasone (1 mg) injection, treatment that will go on each week for another 3 weeks and then another 5 days with glucagon.

2. RESULTS AND DISCUSSIONS

Case no. 1

In this case, althought we obtained good results after all the analises, we could not established the right cause of hypoglycemia and epileptiform episods, because the MRI examination was not performed.

The MRI ecomandation was based on the suspicion of a hipophysal tumor that could be a cause of this type of symptoms.

Case no. 2

Unfortunately, the second day, after a series of strong epileptiform seizures the animal died.

In this case, it is suspected that because an abundant meal of chicken and of the pancreatic formation (insulinoma), the pancreas was overcome and could not perform its function. We tried to correct the glycemia with perfusions glucose, first 5% and then 10%, but because of the high quantity of insulin produced by the pancreatic tumor formation, this could not be maintained at values compared with an

optimal functioning of the nervous system. The animal's nervous system reacted to this deficiency with convulsive episodes, ultimately occurring both hypoglycemic coma and heart congestion and collapse, followed by the death of the animal.

To try to reestablish the balance insulin – glucagon, the hyperglyceminat hormone, glucagon treatment was recommended (commercial product Glucagen), but the fulminant evolution did not gave us any time to have some results.

Case no. 3

On 30.05 the dog had another of loss of balance episode, loss of consciousness and muscle tremors, hypersalivation and postictl confusion; the seizure lasted about 3 minutes and the glycemia was 19 mg/dl. The animal is still under treatment, it received a second glucagon cure and a second series of dexamethasone injections, during this time the glycemia did not drop under 50 mg/dl. The animal also received diprophos once every 3 weeks.

The animal state of health was reevaluated in July and this was very good. The blood biochemical analysis results were within normal limits, the hematologic examination did not show any change and on the ultrasound examination was examined the form and dimensions of the pancreatic formation declared in April. This formation grew very little (0.31 cm) (Fig. 3).

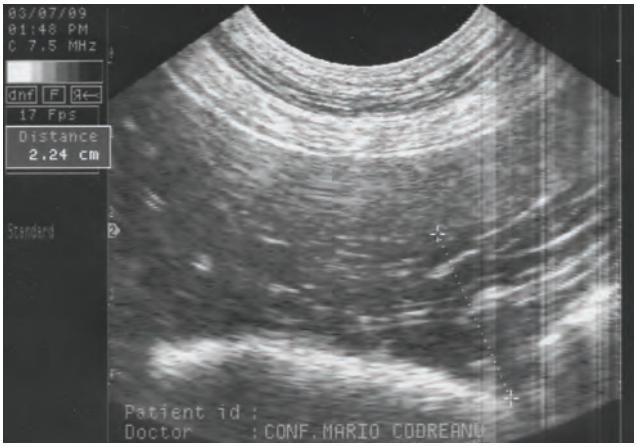

Fig. 3 – The reevluated ultrsound examnation that showed enrargement of the pancreatic lobe with 0.31 cm.

An ultrasound reexamination and also blood parameters reevaluation was recommended in 3 months.

The dog's diet still includes sugar and honey. The anti-inflammatory therapy aims to stimulate the adrenal glands and the default appearance of the hyperglycemia effect, through which glycemia is maintained at values compatible to normal activity of the all the organs and to a satisfactory quality of the animal's life.

3. CONCLUSIONS

3.5. Miscellanous caused hypoglycemia could be a reason of epileptiform episods in dogs.
3.6. The therapy applied in such cases aims to remit the epileptiform episodes by antiepileptic drugs administration as well as fighting against hypoglycemia.
3.7. In such situations, on long term, an important role plays the treatment of primary etiology that provoked hypoglycemia, that is itself a cause of epileptiform episods.
3.8. The pursuance of hypoglycemia is an important desideratum because a possible hypoglicemic coma could have a fatal effect.

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ALLERGIC CONTACT DERMATITIS, IN DOG (CASE REPORT)

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Key words: allergic contact dermatitis, anti-flea collar, dog

SUMMARY

In dogs and cats this type of sensibilization has to be differentiated from irritant dermatitis that is quite difficult taking into account the same substance is simultaneously irritant and allergenic.
Our findings revealed a case of allergic contact dermatitis to anti-flea collar in a Shar pei dog, 1 year old. The neck lesions are present after 4 days since the application of the collar. Clinical anamnetic data and the results of paraclinical tests combined with symptomatic therapy supported a hypersensitivity to the antiparasitic compound of anti-flea collar.

The allergic contact dermatitis is rarely diagnosed in dogs and extremely rarely in cats.In all the cases, this state of hypersensitivity must be differentiated from the irritative dermatitis, which is extremely difficult, considering the fact that a certain substance may be at the same time irritative and allergenic.

We have signalled a case of contact allergy in the anti-flea collar, in a male of the race Shar pei aged 1 year. From the anamnesis it resulted that the lesions developed in about 4 days from the application of the collar, around the back of the neck, beginning with a moderate rash, alopecia, papules at the level of the neck, subsequently the lesions got an eczema-like festering aspect, with intense rash, until self-mutilation.

The differential clinical diagnosis has been done with respect to:alergia/intoleranta alimentara, dermatita atopica, raia sarcoptica, piodermita superficiala Allergy/food intolerance, atopic dermatitis, sarcoptic mange, superficial pyodermitis.

1. MATERIAL AND METHODS

In order to leave out the causes of parasite, mycotic and bacterial nature skin scraping has been carried out, with inoculation on the mediums adequate for bacteria (bullion/nutritional agar) and miceti (Sabouraud glucosis 2%) Moreover, blood samples have been collected for the haematological routine exam (leukocyte formula per panoptical coloured smears MGG).

2. RESULTS AND DISCUSSIONS

By the corroboration of the clinical-anamnesis data with the data of the paraclinical examinations the existence of a hypersensitivity state towards the active substance in the anti-flea collars has been established, complicated with a superficial pyodermitis caused by Staphylococcus intermedius beta haemolytic. In the support the diagnosis of allergy there were also the data of the haematological examination which revealed a moderate basophilia (5%), the blood presenting a dark red aspect.

Moreover, the symptomatic treatment consisting in the removal of the collar, the application of topics on the grounds of ***dichlorhexidine*** gluconate, unguent based on hydrocortisone 2%, along with general antibiotic-therapy, pleaded for the same diagnosis of contact allergy because the lesions improved within 6 days since its establishment.

3. CONCLUSIONS

3.1. The allergic contact dermatitis is a dermatological condition rarely found in dogs and extremely rare in cats;

3.2.. The differential clinical diagnosis is in order especially compared to the atopical dermatitis, allergy/food intolerance, dermatophytoses, ectoparasitoses;

3.3. The allergic contact dermatitis is very difficult to differentiate from the irritative dermatitis by the fact that a certain substance can be at the same time irritant and allergenic.

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FINDINGS REGARDING THE PRURITIC DERMATITIS IN DOG

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Key words: pruritic dermatitis, dog

SUMMARY

Cutaneous pruritus often causes an unpleasant sensation in pet, alarming the owner on a potential systemic or dermatological disorder. The lock of specific features and developing of complications make difficult the confirmation diagnosis in pruritic dermatitis witch needs a systematic and differential approach to identify the primary source of the lesions. This study performed on 89 dogs of different breeds and ages intended etiologic classification of the pruritic dermatitis in dog. The statistics revealed a high incidence of bacterial and ectoparasitic dermatitis by comparison fungal, allergic and other origins of dermatitis (immune, endocrine).

Cutaneous pruritus is the most important cause of discomfort for the pets, often alerting the owner with respect to a possible dermatological and/or systemic condition.Due to the lack of specificity of this symptom and the complications it causes, the veterinary physician often faces diagnosis difficulties, and he she/he has to remove methodically and differentially the possible causes for the identification of the primary aetiology of the existent lesions.

This study aims at separating per etiological criteria the pruritic dermatitis in dogs. The results showed that the most frequent cause of the cutaneous pruritus in dogs was represented by the dermatitis of bacterial origin (pyodermatitis) and those of ectoparasite origin. For the establishment of the aetiology of this symptom, a careful differential diagnosis must be carried out, as a result of the corroboration of the anamnesis with the clinical and paraclinical data.

In case of pruritic dermatitis, most of the specialists recommend the following testing pattern: tests to infirm the ectoparasitism, tests to infirm the bacterial aetiology, tests for the evaluation of the hypersensitivity states, followed by mycological, haematological, biochemical, hystopathological examinations.

1. MATERIAL AND METHODS

A number of 89 dogs have been examined, 47 females and 42 males, aged between 4 months and 8 years, predominantly from the breeds: Boxer, Shar pei, Cocker, Ciobanesc german, Setter, Caniche etc. the pruritus being the clinical sign constantly met, having various aspects: Localized or generalized, of low intensity, up to scratching, sometimes seasonal, sometimes permanent. At the same time as this, lesions of pyodermatitis, of alopecia, of hyper-pigmentation have been signalled.

In order to mention the diagnosis with certitude, several tests have been made:tests infirming the ectoparasitism (direct exam of the cutaneous scraping), tests infirming the bacterial aetiology (smears from coloured lesions Gram, inoculations on adequate mediums, antibiotic-therapy), tests infirming the mycotic aetiology (direct exam of the cutaneous scraping, inoculations on adequate mediums), evaluation tests of the hypersensitivity (intradermic allergic tests with different aeroallergens).

2. RESULTS AND DISCUSSIONS

The evaluation of the cases on the grounds of the aetiological criterion emphasized the fact that in most of the cases the cutaneous pruritus has had a bacterial and ectoparasite origin.

Total animals: 89

The bacterial dermatites (pyodermatites) developed at the same time as the pruritus of various intensities, presented polymorph clinical aspects, being predominantly of the superficial type (papules, crusts, epidermal collarettes, pustules). The most frequently isolated germs have been: Staphylococcus intermedius (71%) and Staphylococcus aureus (29%).

In case of the ectoparasitoses, the demodecia has been diagnosed with the highest incidence, clinically being found both in the localized form (alopecic spots) and the generalised dry form (alopecic diffuse, moderate erythema, accentuated seborea) and wet (pyodemicosis). The pruritus has been constantly found, of various intensities.

The ectoparasitism with fleas and the sarcoptic mange have also been present in some cases. The pruritic dermatites of mycotic origin diagnosed throughout this study have been represented by the dermatophytoses (microsporia, tricophitia) and levuroses (candidosis, malasseziosis), the infections having a superficial location. In all the

cases, the microsporia has been the most frequent dermatomicosis, the lesions being of the alopecic placard type, with weak pruritus.

In the establishment of the aetiological diagnosis, the maximum difficulty has been found in the diagnosis of the dermatitis of allergic nature. The diagnosis has been cumbered by the irrelevance of the clinical laboratory tests, as well as by the constant therapeutic ineffectiveness. The clinical signs have displayed an accentuated polymorphism, from generalized forms to strictly localized forms, insidious, the pruritus being constantly found. In few of the cases (4) the origin of the aetiological agent could not be established.

3. CONCLUSIONS

3.1. The cutaneous pruritus often creates the most serious diagnosis problems in the veterinary dermatology;

3.2. The dermatites of bacterial and ectoparasite origin are the most frequent causes of the cutaneous pruritus in dogs, of which especially noticeable are the staphylococcus pyodermites and the demodicosis;

3.3. The pruritic dermatites of the allergic nature are difficult to diagnose compared to the mycotic ones, as they require a careful corroboration of the clinical-anamnesis data with the results of the intradermic allergic tests;

3.4. The pruritus is responsible in most cases for the appearance of complications sometimes leading to the damaging orientation of the diagnosis.

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THE RESULTS OF THE PARACLINICAL INVESTIGATION IN SOME LIVER DISEASE IN DOGS

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Keywords: liver, disease, dogs

SUMMARY

This work presents clinical and paraclinical investigations by comparison in healthy dogs, dogs with acute hepatitis and with chronic hepatitis. The study was made on 34 dogs with clinical signs of acute or chronic hepatitis. The results of our investigations revealed the necessity of the paraclinical exams.

Considering the high frequency of the hepatobiliary disorders in dogs, by this paper I aimed at accomplishing a study regarding the significance of the laboratory exams data, which, corroborated with the clinical signs, as well as other means of investigation of the liver, should help us in the establishment of a precise diagnosis of the hepatobiliary disorders in order to adopt an adequate therapeutic behaviour.

The liver has a high storage capacity, functional reserves and regenerative abilities, therefore, the injuries on the hepatic cell must be severe or must be associated with a cholestasis before the clinical signs should be obvious and the routine laboratory tests should express a hepatic condition. The hepatic dysfunction is not traced before the moment when more than 55% of the liver functional mass is lost.

For dogs, of special importance are the acute and chronic hepatitis, leading to the appearance of the syndrome of extremely serious hepatic insufficiency, sometimes jeopardizing the animals' lives.

1. MATERIAL AND METHODS

The investigations of this paper have been carried out in the period July 2007 – July 2008 on a number of 34 cases in dogs.

The study has been carried out on dogs belonging to different breeds, of different ages, which presented acute or chronic hepatitis, for the paraclinical evaluation, for the establishment of the best therapeutic approach.

The laboratory determinations have been carried out in the specialised laboratory within the Faculty of Veterinary Medicine Bucharest with the help of the apparatus Reflotron IV.

2. RESULTS AND DISCUSSIONS

The study material consisted in the cases presented in various veterinary practices in Bucharest and diagnosed light hepatic insufficiency or severe hepatic insufficiency.

Presentation of the cases with hepatic disorders

Table 1

Presentation of liver diseases cases

No. animals	Liver diseases
8 dogs 4 males 2-5 years 4 females 2-6 years	light hepatic insufficiency
15 dogs 7 males 7-12 years 8 females 7-11 years	severe hepatic insufficiency
Total - 23 dogs	

THE CLINICAL DIAGNOSIS OF THE HEPATOPATHIES IN DOGS

1.1. The clinical diagnosis in the light hepatic insufficiency

In the clinical examination we notice:

- Apparently normally coloured mucosas;
- Light hepatic sensitivity;
- Reduction of the joy, capricious appetite, tendency to lose weight;
- Biliary vomiting.

In table no.are presented the data of the paraclinical examination.

Table no 1

The values of the activity of serum transaminases, bilirubinemia and glycaemia in animals with light hepatic insufficiency compared to the ones recorded in the healthy animals (dosages at Reflotron).

Diagnosis	No. animals	Value of the serum transaminase activity (U/l)			Bilirubinemia mg/dl	Glycaemia mg/dl
		GGT	GPT	GOT		
Healthy	10	3-5	37-40	36-40	0.40-0.60	64-88
Light hepatic insufficiency	4	8-11	42-52	68-71	0.70-1.25	91-95

The normal value of the glycaemia is **60-90 mg/dl.**

The clinical diagnosis in the severe hepatic insufficiency.

Of the anamnesis data it results:

- anorexia ;
- Lost of weight;
- Decoloured faeces with fetid smell;
- Diarrhoea alternating with constipation;
- Apathy, depression or excitement;
- Green vomiting;

Clinically, we noticed:

- Icterus;
- Sad facies;
- Dehydration;
- Fever 38˚C;
- Hepatomegaly, the liver is sensitive and tough;
- Bradicardy.

In the echographic examination, a hepatomegaly is noticed.

Table no. 2

The values of the activity of serum transaminases and of the alkaline phosphatasis in animals with severe hepatic insufficiency and in the clinically healthy animals (dosages at Reflotron).

Diagnosis	No. animals	Value of the transaminases activity			Alkaline phosphatasis U/l
		ALAT U/l	ASAT U/l	GGT U/l	
Healthy	10	37-39	38-40	4-5	145-190
Severe hepatic insufficiency	15	75-87	84-89	7-9	190-244

From the data of table no. 2 it results that the animals with severe hepatic insufficiency present an increase of the activity of serum transaminases and of the alkaline phosphatasis.

The high values of the transaminases activity and of the alkaline phosphatasis play a big part in the establishment of the diagnosis of severe hepatic insufficiency.

Table no. 3

The values of the bilirubinemia, cholesterolemia and glycaemia in healthy animals and in clinically healthy animals (dosages at Reflotron)

Diagnosis	No. animals	Bilirubinemia Mg/dl	Cholesterolemia	Glycaemia mg/dl
Healthy	10	0.35-0.60	110-435	50-90
severe hepatic insufficiency	15	1.10-2,30	88-104	125-170

According to the data of table no.it results hyperbilirubinemy, hypoglycaemia, hypercholesterolemia.Hypercholesterolemia denotes a serious alteration of the liver.There were also recordedThe VSH was moderately increased, the coagulation times increased because of the reduction of the synthesis in coagulation factors by the liver, especially of the protrombine, hyperglycaemia states were also recorded. The plasmatic proteins are low (hypoproteinemy). The urine is over coloured with albuminuria, glicosurie, cetonury, bilirubinuria

The paraclinical diagnosis confirms the clinical diagnosis of serious hepatic insufficiency and it is based on:hyperbilirubinemy, increased

enzymatic activity (alkaline phosphatase and serum transaminase), low hematocrit, hypocholesterolemy, hypoproteinemy).

Table no. 4

The synthetic presentation of the laboratory examinations data in the dogs with severe hepatic insufficiency compared to the data obtained in healthy dogs (dosages by the use of Reflotron).

Determinations followed	Clinically healthy animals		Animals with severe hepatic insufficiency	
	No. cases	Values	No. cases	Values
ASAT (U/l)	10	38-40	15	84-89
ALAT (U/l)	10	37-40	15	75-87
GGT (U/l)	10	4-5	15	7-9
PAL (U/l)	10	145-190	15	190-244
Bilirubinemia (mg/dl)	10	0,35-0,60	15	1,10-2,30
Cholesterolemia (mg/dl)	10	110-435	15	88-104
Glycaemia (mg/dl)	10	50-90	15	125-170

The data from the table above reveal an increase of the enzymatic activity of the serum transaminases and alkaline phosphatases; the increase of the bilirubinemy, cholesterolemy, glycaemia; in animals with severe hepatic insufficiency compared to clinically healthy animals.

3. CONCLUSIONS

In **dogs with light hepatic insufficiency,** were noticed the following changes of some sanguine biochemical parameters (dosages carried out at Reflotron and in the Ecom 6122 photometer).

The serum transaminases present increased activity in the dogs with light hepatic isufficiency compared to the animals in the placebo group. The values of the ASAT activity in the dogs with light hepatic insufficiency ranged from 68-71 U/l, those of ALAT between 42-51 U/l, and those of GGT between 8-11 U/l. They signify the presence of hepatic lesions without obvious clinical displays. The increase of the serum transaminases activity in the animals with light hepatic insufficiency is not as high as the increase of the serum transaminases activity in the hepatitis animals.

The values of the serum bilirubin ranged from 0.75-1.25 mg/dl, compared to 0.40-0.60 mg/dl in healthy animals, so higher values in the animals with light hepatic insufficiency.These increases, although reduced, are explained by a beginning of hypersecretion of bilirubin.

The glycaemia was slightly changed compared to the values met in the following group:in the dogs with light hepatic insufficiency and values ranging between 64-88mg/dl in the clinically healthy animals.

In **dogs with severe hepatic insufficiency,** were noticed the following changes after the dosages at Reflotron and in the Ecom 6122 photometer.

The activity of the serum transaminases (ASAT, ALAT,GGT) and the activity PAL were increased in the dogs with severe hepatic insufficiency, the highest values were noticed in the case of ALAT and PAL: the value of the ALAT activity ranged between 75-85 U/l in the sick animals compared to 37-39 U/l in the healthy ones; the PAL activity had values between 190-244 U/l compared to 145-190 U/l in the clinically healthy ones. The values of the ASAT activity ranged from 84-89 U/l in the sick animals, compared to 38-40 U/l in the healthy ones. The values of the GGT activity ranged from 7-9 U/l in the sick animals, compared to 4-5 U/l in the healthy ones. The increase of the serum transaminases activities (ALAT and ASAT) and the alkaline phosphatase may appear by a high enzymatic synthesis, by a high enzymatic degradation or by the stimulation of the activity of these enzymes without the increase of the molecule number.

The bilirubinemy has been highly increased: 1,10-2,30 mg/dl 1.0-2.0 mg/dl in the animals with severe hepatic insufficiency compared to 0.35-0.60 mg/dl in the healthy ones. In the severe hepatic insufficiency, the massive increase of the bilirubinemy is owed to a hepatocellular disease , acute or chronic. In the case of these disorders, the tumefaction of the hepatocytes may obstruct the biliary canicules, determining intrahepatic colestasis and the regurgitation of the bilirubinemy conjugated in the blood.

The glycaemia was higher in the dogs with severe hepatic insufficiency (125 - 170 mg/dl) compared to the placebo group (50-90 mg/dl), which proves a disorder of the glucose-regulating function of the liver by the insufficiency of the conversion in glycogen of the circulating glucose.

The cholesterolemy was lowered in the dogs with severe hepatic insufficiency (88-104 mg/dl) compared to the clinically healthy ones (110-435 mg/dl) which proves a serious diffuse alteration of the hepatic parenchyma.

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THE RESULTS OF THE CLINICAL AND PARACLINICAL INVESTIGATIONS IN ICTERUS IN DOGS

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Keywors: icterus, dogs, investigations

SUMMARY

There are many different causes for icterus, but they can be divided into three categories based on where they start-before, in, or after the liver (prehepatic, hepatic and post-hepatic). When bilirubin begins its life cycle, it cannot be dissolved in water. The liver changes it so that it is soluble in water. These two types of bilirubin are called unconjugated (insoluble) and conjugated (soluble). Blood tests can easily distinguish between these two types of bilirubin.

In the early stage of the icterus by obstruction, most of the functional tests usually provide negative data, except for the dosage of alkaline phosphatase which is high.

In case of extended icterus, in which if the icterus had initially been mechanical, in a more advanced stage secondary hepatic parenchymal alterations are associated to it (hepatomegaly, acute yellow atrophy) which make the tests positive. The tendency of some functional samples to become negative, although previously being positive and vice-versa, initially negative tests becoming positive may provide useful information regarding the pathogeny of the icterus, in its evolution.

Although all the degenerative hepatic diseases, inflammatory or functional, primary or secondary, specific or non-specific, very frequent are the discretion of the clinical displays and the difficulties of a paraclinical diagnosis of high precision for these affections, often hinders their discovery until the most advanced stages or by necroscopic examination.

1. MATERIAL AND METHODS

The investigations of this paper have been carried out in the period December 2008 – May 2009 on a number of 16 cases in dogs.

The study has been carried out on dogs belonging to different breeds, of different ages, which presented icterus, for the paraclinical evaluation, for the establishment of the best therapeutic approach.

The laboratory tests have been carried out with the help of the apparatus Reflotron IV.

2. RESULTS AND DISCUSSIONS

The study material consisted in the cases presented in various veterinary practices in Bucharest and diagnosed with icterus.

Table 1

Presentation of liver diseases cases

No. animals	Liver diseases
16 dogs 8 males 2-12 years 8 females 2-11 years	Icterus

THE CLINICAL AND PARACLINICAL DIAGNOSIS IN THE ICTERUS SYNDROME

The anamnesis data showed:

- Progressive loss of weight;
- Constipation with faeces of a yellowish-white colour;
- Vomiting;
- Capricious appetite;

The clinical examination showed:

- Icteric coloration of the apparent teguments and mucosas;
- Fever 38˚C;

Table 2

The values of the bilirubinemia, cholesterolemia, the value of the alkaline phosphatase activity and the gamma glutamil-transpherase

Diagnosis	No. animals	Cholesterolemia mg/dl	Bilirubinemia mg/dl	PAL U/l	GGT U/l
Healthy	10	102-442	0.40-0.60	150-165	4-5
Cholestasis	3	485-560	1.68-4.20	216-350	7-11

The laboratory diagnosis considers:

- The hyperbilirubinemia;

- The increase of the alkaline phosphatase activity (PAL);
- The increase of the GGT activity;
- Hypercholesterolemia.

The normal value of the alkaline phosphatase activity:**<190 U/l. <190 U/l.**

The normal value of the bilirubinemia: **<0,61 mg/dl.**

The normal value of the cholesterolemia: **<104-440 mg/dl.**

Table 3

The synthesis presentation of the laboratory examinations data in the dogs with cholestasis compared to the data obtained from healthy dogs

Determinations followed	**Clinically healthy animals**		**Animals with cholestasis**	
	No. cases	**Values**	**No. cases**	**Values**
GGT (U/l)	10	4-5	3	7-11
PAL (U/l)	10	150-165	3	216-350
Cholesterolemia (mg/dl)	10	102-442	3	485-560
Bilirubinemia (mg/dl)	10	0.40-0.62	3	5-9

The data in this table shows an increase of the GGT and PAL activity as well as the increase of cholesterolemia and bilirubinemia.

In the confirmation of the obstructive nature of the hepatic affections it is important that cholesterolemia and bilirubinemia should increase rapidly.

CONCLUSIONS

The activity of the serum transaminases was increased in the sick animals: the values of the GGT activity in the sick animals was l 7-11 U/l compared to 4-5 U/l in the healthy ones; the value of the PAL activity increased in 216-350 U/l in the sick animals compared to 150-165 U/l in the healthy ones; the cholesterolemia was 485-560 mg/dl in the sick animals compared to 102-442 mg/dl in the clinically healthy ones; bilirubinemia was 5-9 mg/dl in the sick animals compared to 0,40-0,60 mg/dl in the clinically healthy ones.

These values obtained, namely the fast increase of the cholesterolemia, bilirubinemia and GGT activity, confirm the obstructive nature of the biliary ways.

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STUDY CONCERNING BLOOD PRESSURE IN CLINICALLY HEALTHY AND CONSCIOUS CATS MEASURED BY OSCILLOMETRIC METHOD

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Key words: arterial blood pressure, cat

SUMMARY

The aims of this study were to determine the arterial blood pressure of clinically healthy cats and to set up reference values for clinical application. The arterial blood pressure was measured by oscillometric method in 159 clinically healthy cats. Cats were between 11 months and 14 years old, and the mean was 5.96 ± 3.48 years and the group was consisted of cats of different breeds. The mean systolic blood pressure (SBP) was 124,7± 8,54 mmHg. The diastolic blood pressure (DBP) and the mean arterial blood pressure (MBP) were 75,4 ± 10,08 mmHg, 91,9 ± 8,45 mmHg respectively. Feline SBP between 113,7 -135,6 mmHg and DBP between 61,6 - 89,2 mmHg are indicative of normotension. In the clinical setting, SBP/DBP values that are higher than 141,7/95,5 mmHg are strongly suggestive for arterial hypertension , and the decrease of SBP/DBP below 107/55 mmHg indicates the tendency to hypotension. In cats of this study, there is a slight but significant correlation, between age and systolic, diastolic and mean blood pressure.

Blood pressure (BP) may be measured with direct or indirect methods. Although the direct measurement is the most accurate method for blood pressure evaluation, it is generally not applicable in clinical practice as they may require anesthesia or sedation. The indirect methods may be easily applied under clinical conditions, require fewer constraints and may be easily applied from a technical viewpoint. Among these, the *Doppler* and oscillometric methods fit best cats and dogs (Brown et al., 2007; Egner Beate, 2003). Although in human medicine blood pressure measurement has become, for a long time, a frequently used method of investigation, in veterinary medicine it has been not routinely performed in clinically practice. The blood pressure limits in healthy cats have not been very accurately established so far, because the studies proved that there are significant differences between the BP values measured with the indirect methods. The goal of this study is to evaluate the blood pressure measured with the oscillometric method in clinically healthy cats in the clinical setting. The objectives were: the establishment of reference BP values measured with the oscillometric method in healthy cats; the establishment of the limits

from where cats may be considered hypotensive or hypertensive; assessment of the correlations between age, body condition and blood pressure in clinically healthy cats.

MATERIAL AND METHOD

The study was carried out on 159 outpatient clinically healthy cats, which were brought into Timisoara Universitary Veterinary Clinics. Cats were between 11 months and 14 years old, and the mean was 5.96 ± 3.48 years. The group was consisted of cats of different breeds, and the proportion between sexes was approximately 1:1, respectively 80 females and 79 males. Body weight was comprised between 2 and 8 kg, with a mean of 4.48 ± 1.11 kg. The body condition was assessed with a system of clinical score, as follows: 1 - very thin; 2 - mildly underweight; 3 - fit body condition; 4 - mildly overweight; 5 - obese. The cats were assessed by physical examinations, clinical signs and routine blood profile to exclude renal disease or other disorders.

Blood pressure was determined by oscillometric method, with the *Cardell Veterinary Monitor 9401* device, which measures systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MBP) and pulse frequency. Blood pressure measurement was performed at the thoracic limb, at median artery level, with cuffs width of about 30-40% of the forearm circumference. The measurement (BP) was performed in the presence of cat owner, after a 15-minute accommodation of the cat with the area and with the measurement equipment. BP evaluation was performed with cats in a comfortable position. The cats that were too stressed were held by their owners in their arms, and the thoracic member was maintained at heart height level. For each cat, the systemic blood pressure value was calculated as the mean of five consecutive measurements.

2. RESULTS AND DISCUSSIONS

The analysis of variance between the blood pressure values obtained at the five consecutive measurements showed that there are not significant differences regarding SBP ($F = 0.43$; $p = 0.78$), DBP ($F = 2.07$; $p = 0.08$), MBP ($F = 1.65$; $p = 0.15$) and pulse frequency ($F = 0.05$; $p = 0.99$). However, we may notice that between the first and the fifth measurements, the mean BP values decrease, but the differences do not overtake 3 mmHg and are not statistically significant (table 1).

Table 1

Mean values of blood pressure and pulse frequency in five consecutive measurements

Number of measurement	n	SBP (mmHg)	DBP (mmHg)	MBP (mmHg)	Pulse frequency
1	159	124,6 ± 10,2	77,31 ±11,5	93,0 ± 9,3	134,6 ± 22,3
2	159	125,3 ± 10,9	76,6 ± 12,1	92,8 ± 9,9	134,4 ± 24,6
3	159	124,7 ± 11,1	74,3 ± 13,0	91,1 ± 10,7	134,2 ± 23,6
4	159	125,1 ± 10,4	74,9 ± 12,3	91,6 ± 10,5	135,3 ± 22,9
5	159	123,9 ± 9,6	74,2 ± 11,8	90,7 ± 9,8	134,6 ± 22,3

Note: SBP-systolic blood pressure; DBP-diastolic blood pressure; MBP-mean arterial blood pressure;

Each cat's blood pressure was calculated as arithmetic mean of the five measurements and was used in the statistical processing to establish the mean BP values of the clinically healthy cats (table 2).

Table 2

Descriptive statistics of the blood pressure and pulse frequency values calculated as mean of five consecutive measurements

Variable	n	Mean	Standard deviation	Standard Error Mean
SBP (mmHg)	159	124,7	8,54	0,67
DBP (mmHg)	159	75,4	10,08	0,79
MBP (mmHg)	159	91,9	8,45	0,67
Pulse frequency	159	134,8	19,95	1,58

Note: SBP-systolic blood pressure; DBP-diastolic blood pressure; MBP-mean arterial blood pressure

The mean BP values of the cats in this study are similar with the ones obtained, with the oscillometric method, by other authors (Bodey and Sansom, 1998; Mishina et. al., 1998), but are smaller than the values obtained with the *Doppler* method (Lin et al., 2006; Sparkes et. al., 1999).

Although there are several studies regarding cat blood pressure, we have not had yet an agreement regarding BP limits in healthy cats. Only a few studies attempted to calculate these limits according to a statistical method, which was applied in the calculation of variation limits of BP in human. This method considered the mean ± 1,282 standard deviations (Lin et al., 2006; Mishina et. al., 1998; Sparkes et. al., 1999). Starting with this algorithm, the BP limits of the cats in this study are presented in table 3.

Table 3

Variation limits of blood pressure and pulse frequency in healthy cats

Variable	n	Variation limits	
		Minim	Maxim
SBP (mmHg)	159	113,7	135,6
DBP (mmHg)	159	61,6	89,2
MBP (mmHg)	159	81,1	102,7
Pulse frequency	159	109,3	160

Note: SBP-systolic blood pressure; DBP-diastolic blood pressure; MBP-mean arterial blood pressure;

According to the statistical method, the limit between normotension and hypertension may be established by adding two standard deviations to the mean, and the limit between normotension and hypotension by subtracting two standard deviations from the mean (Lin et al., 2006; Mishina et. al., 1998; Sparkes et. al., 1999). 95% of the BP values measured may be found within the interval mean ± two standard deviations. By applying the same algorithm in the blood pressure values obtained in this study, the minimal values from which we may take into consideration hypertension are SBP/DBP = 141.7/95.5 mmHg. In the other extreme, the limits between normotension and hypotension were SBP/DBP = 107/55 mmHg (table 4).

Table 4

The limits between normotension and hypotension/hypertension set with the statistical method

Variable	Algorithm	Limits	
		Hypotension	Hypertension
SBP (mmHg)	**x ± 2s**	< 107	>141,7
DBP (mmHg)	**x ± 2s**	< 55,3	> 95,5
MBP (mmHg)	**x ± 2s**	< 75	> 108,8

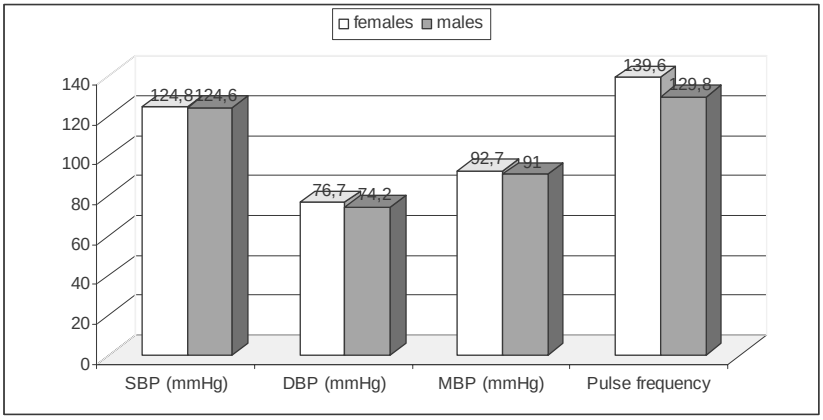
Note: x = mean; s = standard deviation

The results are similar with the ones obtained in a study performed on 60 clinically healthy cats, where, according to the same formula, the inferior hypertension limit was 140 mmHg for SBP and 95 mmHg for DBP (Mishina et al., 1998). Also, Curtet et al. quoted by Rosane Jepson et al. recommend the utilization of the values 109 and 137 mmHg as minimal and maximal variation limits of SBP measured with the oscillometric method in clinically healthy cats (Rosane Jepson, 2005).

In literature, the criteria for feline hypertension published so far range from 141 mmHg to 200 mmHg. Only in a small number of studies the values were established through statistical analysis, but the results

were very different (141, 161, 200 mmHg), because the differences between the mean SBP values were considerable (Lin et al., 2006; Mishina et. al., 1998; Sparkes et. al., 1999).

There are not significant differences (p>0.05) between males and females regarding SBP, DBP and MBP, although pulse frequency was significantly higher in females than in males (fig. 1).



Note: SBP-systolic blood pressure; DBP-diastolic blood pressure; MBP- mean arterial blood pressure

Fig. 1. Mean values of blood pressure in males and females cats

The correlation between age and blood pressure was a slight one, but significant in the case of SBP, DBP or MBP. On the contrary, there is not a significant correlation between pulse frequency and age (r = 0.13; p >0,05). The analysis of regression showed that blood pressure increases with the age. So, the equation of regression line proved that SBP increased with 0.7 mmHg/year (fig. 2), and DBP increased with 0,8 mmHg/year (fig. 3).

Table 5

Correlations between age, blood pressure, body condition score and pulse frequency in clinically healthy cats

Variable		**Age** (months)	**SBP** (mmHg)	**DBP** (mmHg)	**MBP** (mmHg)	**Pulse frequency**
Age (months)	**Pearson Corelation**	1,000	0,315**	0,363**	0,394**	0,135
	p	-	0,000	0,000	0,000	0,090
	n	159	159	159	159	159
Body condition score	**Pearson Corelation**	0,296**	0,043	0,143	0,128	-0,258**
	p	0,000	0,592	0,093	0,108	0,001
	n	159	159	159	159	159
****Correlation is significant at the 0.01 level (2-tailed)**						

Note: SBP-systolic blood pressure; DBP-diastolic blood pressure; MBP-mean arterial blood pressure

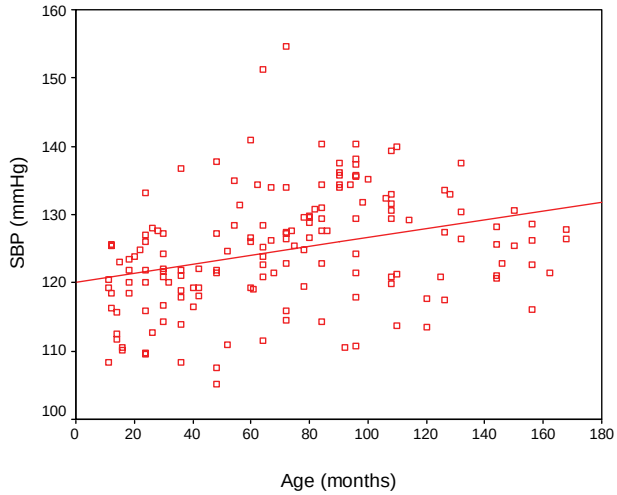


Fig. 2. Liniar regression between age and SBP values (Y=122 + 0,0685X; r = 0,31; p < 0,0001)

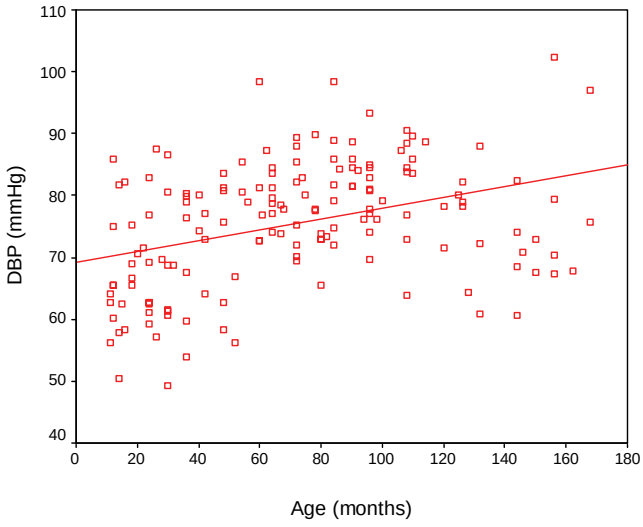


Fig. 3. Liniar regression between age and DBP values (Y=64,9 + 0,077X; r = 0,36; p < 0,0001)

The previous studies regarding the correlation between age and blood pressure led to contradictory results. Bodey and Sansom, in a study performed on 203 cats, where they determined blood pressure with the oscillometric method, observed that TAS, TAD and pulse frequency were significantly higher in the cats older than 11 years old

compared with the ones younger than 11 years old. (Bodey and Sansom,1998). Also, the researches performed by Mishina et al. on 60 clinically healthy cats, showed a significantly positive correlation between age and DBP, MBP respectively (Mishina et al., 1998). These results were contradicted by the result of the researches performed by Kobayashi et al. and Sparkes et al., who concluded that there was not a positive correlation between age and blood pressure value in clinically healthy cats (Kobayashi et al., 1990 and Sparkes et al., 1999). The difference between these studies consists in the fact that, in the last two, BP was measured with the *Doppler* method, which cannot evaluate DBP and MBP values.

The correlation between BP and the body condition score, was insignificant regarding SBP ($r = 0.043$; $p = 59$), and DBP as well ($r = 0.14$; $p = 0.73$), or MBP ($r = 0.11$; $p = 108$) (table 5). Also there were not significant differences of SBP, DBP and MBP values between the cat groups differentiated according to the clinical score.

In human, obesity represents an important risk factor in the apparition of hypertension. Some studies proved that excessive fatness could lead to the increase of blood pressure in dogs, too, while, in cats, the existence of a positive and significant correlation between blood pressure and maintenance status was not proved (Bodey and Sansom, 1998; Bodey and Michell, 1996; Lin et al., 2006).

3. CONCLUSIONS

3.1. In cats of this study, normotension can be defined as SBP between 113,7 -135,6 mmHg and DBP between 61,6 -89,2 mmHg, respectively.

3.2. In the clinical setting, SBP/DBP values that are higher than 141,7/95,5 mmHg are strongly suggestive for arterial hypertension, and the decrease of SBP/DBP below 107/55,3 mmHg indicates the tendency to hypotension.

3.3. Blood pressure does not record significant differences between males and females.

3.4. In cats of this study, there is a slight but significant correlation, between age and systolic, diastolic and mean blood pressure.

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RAPID ACCESS SYSTEM OF BLOOD DONORS IN VETERINARY EMERGENCY MEDICINE

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Veterinary emergency medicine, animal blood-bank, veterinary blood transfusion, dog blood donors, cat blood donors

SUMMARY

The transfusion technology for companion animals developed during the nineteenth century, and the request for blood or blood products increased over the last several years. Transfusion can be life-saving in some situations, such as massive blood loss due to trauma, sever anaemia or thrombocytopenia caused by a blood disease, or can be used to replace blood loss during surgery. In this paper we present our approach in creation of the fist animal blood bank in Romania to meet the needs of the field veterinarians and most of their patients. Our purpose is to provide veterinarians with safe blood products, transfusion supplies and knowledge about transfusion. Our goal is to provide the best in patient care, in partnership with veterinarians. This full-service blood bank will provide blood components and supplies for transfusions to veterinary clinics. The program is focused on providing: clinical efficacy use of compatible blood components for patient needs, professional consultations by specialists, patient safety, donor protection against environmental exposure to blood-borne pathogens and control, donor compatibility (our donors must have universally compatible blood type), product availability for critical emergencies through local clinics that stock blood products.

Blood transfusion, the established therapeutic method for anaemic human patients, has recently become more used in veterinary medicine. For companion animals, the necessary transfusion technology and the use of blood or blood products increased over the last several years.

Transfusion can be life-saving in some situations, such as massive blood loss due to trauma, sever anaemia or thrombocytopenia caused by a blood disease, or can be used to replace blood loss during surgery.

A single unit of blood can potentially be divided into three components (red blood cells, plasma and platelets), so, when appropriate, they can then be used separately, getting maximum use from each donated unit of blood and reducing the risk of side effects in the recipient transfusing him just the needed elements. In the present this is routine in human medicine and start to be available in canine medicine, but currently remains rare in feline medicine.

Multiple transfusions can be a problem. Despite the fact that the donor and recipient may be compatible initially, the recipient's immune

system may build up sensitivity to a specific donor. Thus, before each transfusion, the recipient mast be check for cross-match, to avoid the acquired sensitivity under preliminary transfusions. [2].

Transfusion side-reactions may be prevented following the standard protocol for blood transfusion: cross-checking for blood types, healthy blood donor submitted previously to specific tests, to prevent infections or spread of diseases, and appropriate storage of harvested blood.

Dogs have eight different blood groups; labelled as DEA (dog erythrocyte antigen) 1 to 8. Dogs that are DEA 1.1 negative are considered universal donors [2].

In cats there are three major blood groups: A, B and AB. Cats do not have a universal donor; therefore, it is especially important that donor and recipient are cross-matching. It is generally accepted that blood transfusions are more difficult in cats than in dogs. Studies looking to the indications and success of feline blood transfusions suggest that the most common reasons for blood transfusion in cats in Germany and the USA are: anaemia caused by blood loss (27% - 52%), bone marrow failure (38%), anaemia caused by chronic renal failure (20%), haemolytic anaemia (10% - 14%) [1].

Some veterinarians maintain registers of owners who have signalled their willingness to allow their cats to be used as blood donors and this can be very helpful, but even so it is not always easy to find a compatible cat who is available to be brought into the practice immediately and who has not been fed (so he/she can be sedated), and then, running the necessary pre-collection health and infectious disease tests, add additional time and complexity to the situation. Taking all this into consideration, feline blood banks would provide an easily accessible source of safe, pre-tested blood of known blood type, and would make blood transfusion much more practical for veterinary practices. Despite the benefits, collection from pet-owned cats does involve some risk to the cat, and the raft of pre-collection tests required might limit the number of owners willing to ”volunteer” their cats, as well as adding significantly to the financial cost of each unit of blood.

As with human blood donors, animal donors are tested to make sure on blood quality and no infectious disease is present before blood is drawn. Donors must meet weight requirements: 5kg for cats, and 25kg for dogs. Fluid is replaced after blood‘s draw, and the body cover by producing new red blood cells. Also, similar to human donations, there must be a resting period of at least two months before blood is collected again [2].

Private veterinarians sometimes use their pet dogs or cats as blood donors when emergencies arise. Therefore, we are in the process of creating the fist animal blood bank in Romania to meet the needs of the field veterinarians and of their patients.

Our purpose is to provide veterinarians with safe blood products, transfusion supplies and knowledges about transfusion. We are dedicated to our partnership with veterinarians to provide the best in patient care. This full-service blood bank will provide blood components and supplies for transfusions to veterinary clinics. The program is focused on providing: clinical efficacy use of compatible blood components as patient need, professional consultations by specialists, patient safety, donor protection against environmental exposure to blood-borne pathogens and control, recipient compatibility (our donors must have universally compatible blood type), product availability for critical emergencies through local clinics that stock blood products.

On the www.magazinveterinar.ro website, educational materials will be provide in order to support veterinarians and especially pet owners understand the importance of "volunteering" animals to donate blood and, therefore, save other pets' lives. The donors and their owners will be registered into our database which will be accessed every time when the veterinarian or the clinic is needing blood products (Fig 1).

Donors should be checked and approved by a veterinary surgeon. They must be healthy and able to donate blood in order to minimize the risks of donations. An ideal blood donor is a friendly, healthy, clinically normal pet and, if female, not pregnant or nursing. Donors should be immunized (but no sooner than 10-14 days prior donation.

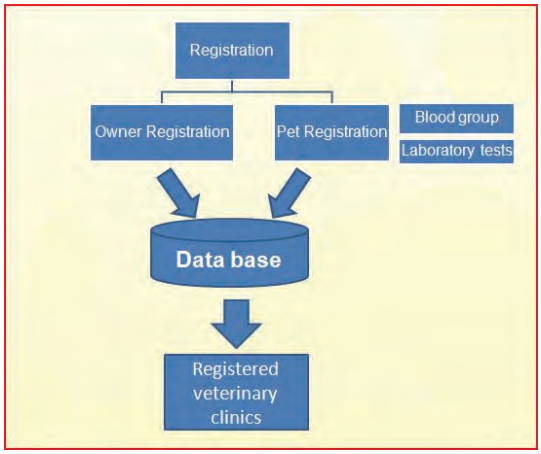

Fig. 1 The blood bank data flow scheme

Blood can be collected from unsedated (if they are cooperating), sedated or anesthetized pets, if necessary. Cats usually need sedation or general anaesthesia. Blood is collected in human blood bags or syringes with anticoagulant. A large and accessible vein is needed: usually jugular vein or, sometimes, the cephalic vein.

A standard blood donation in dogs is no more than 450ml ("a canine unit"), and it can be safely obtained from a 25 kg dog. The cats usually donate 11-13 ml/kg. Repeated blood donations in a relatively short period of time can lead to anaemia and should be avoided unless absolutely necessary. Therefore, after a performed donation, recorded in our database, the donor will not be called for another donation before 2 - 3 months.

In the future our goal is to organize a national veterinary blood bank equipped with diagnosis laboratories for blood-borne diseases. Unfortunately the high costs limit the implementation of the program, but, with the financial support of the veterinary organizations and companies this project could become reality in a very short time.

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IN VITRO EFFECT OF ROMPARASECT FORTE

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Key words - ROMPARASECT FORTE insects, acari ectoparasites.

SUMMARY

The research was done using the product ROMPARASECT FORTE, for external use, produced by ROMVAC COMPANY S.A. The product is marketed as watery solution 2ml/liter of water and is used against acari species and insect ectoparasites. The research was done in Petri plates with diameter of 4.6 mm, in which round sheets of papers imbibed with 0.5 ml ROMPARASECT FORTE. Each plate was populated with 10 specimens of the studied species of ectoparasites. The efficacy of the product was determined against the following acari species – *Psoroptes cuniculi, Psoroptes bovis, Chorioptes bovis, Ripicephalus bursa, Dermanyssus gallinae* and insects – *Haematopinus suis, Linognathus stenopsis, Linognathus vituli, Damalinia bovis, Damalinia caprae, Menopon gallinae* and *Goniodes meleagridis.* After 2 hours of contact the product was 100% efficient against *Psoroptes bovis, Dermanyssus gallinae, Linognathus stenopsis, Linognathus vituli, Haematopinus suis, Damalinia bovis, Damalinia caprae, Menopon gallinae* and *Goniodes meleagridis*. After 2 hours of contact the product was 70% efficient against the tick *Ripicephalus*, 80% against *Psoroptes cuniculi* and 90% against *Chorioptes bovis*.

The therapy and prophylaxis of ectoparasitic infestations in mammalians and birds had available during the recent years a wide range of antihelminthic products which displayed spectacular progresses. In the fight to control the ectoparasitic infestations produced by mites and insects the progress is less obvious against the mange. The products used to control the ectoparasitoses observed in animals were efficient but residues were thereafter detected in animal products – meat, milk etc. (Şuteu I., N. Dulceanu, 2001).

A representative pharmacological category used to control the acarioses and entomoses are the pyrethroid substances. They are synthetic products analogue to the vegetal pyrethrins, but they are much more active and stable (Coman Sofia, 2004).

The pyrethroid substances act by contact and they have neurotoxic effects. They induce in arthropods – acari and insects – a state of hyper excitation followed by paralysis (the "knock down" effect). Many compounds from this group are currently available worldwide, such as deltamethrine, flumethrine, cypermethrine, cyalothrine, permetrine etc. (Cosoroabă I., 2000; Coman Sofia, 2004; Şuteu I., N. Dulceanu, 2001).

The pyrethroid substances are acaricide substances used to control ectoparasitoses in the poultry reared in intensive system due to its low toxicity and due to minimal residues which they leave in the eggs and meat (Coman Sofia et al., 2002; Coman Sofia et al., 2005).

The *in vitro* research which used various acaricide substances against the tick Dermanyssus gallinae showed that the product ROMPARASECT manufactured by ROMVAC COMPANY S.A. as watery emulsion 1 ml/litre of water was 90.4% efficient after 30 minutes of contact, 94.1% efficient after 60 minutes, 95.5% efficient after 2 hours and 100% efficient after 24 hours of contact (Coman Sofia et al., 2005). The results obtained with the product Butox (Deltamethrine) in concentration of 1.7 ml/litre of water were much lower, the efficiency being of 51% after 30 minutes of contact, 65.9% after 60 minutes, 70.5% after 2 hours and 86.4% after 24 hours of contact.

Cernea et al. (2005) reported a 90% efficacy of Diazinon in concentration of 2 ml/litre of water after 2 hours of contact with *Psoroptes cuniculi*, 96.6% after 6 hours and 100% after 12 hours of contact. The product Permethrin, 2 ml/litre of water, was 56.6% efficient after 2 hours, 66.6% efficient after 6 hours and 100% efficient after 12 hours (Colles G.C., K.A. Stafford, 1999). The purpose of this research was to evaluate the *in vitro* effect of ROMPARASECT FORTE for external use, watery solution 2ml/liter of water against the species of acari and ectoparasitic insects infesting the houses and paddocks for animals, the equipment and tools. The product is used for disinsection and desacarition.

1. MATERIAL AND METHODS

The research was done by the department of Parasitology and parasitic diseases of the Faculty of Veterinary Medicine from Spiru Haret University.

Biological material. Species of ectoparasitic acari

The acari *Psoroptes cuniculi* was collected from a rabbit farm. The rabbits were diagnosed with auricular mange after the examination of the crusts harvested from the external auditive duct. *Psoroptes bovis* and *Chorioptes bovis* were obtained from a cattle farm where the animals displayed symptoms of mange disease The ixodid ticks were harvested from a dog brought to the clinic of the faculty; a large number of *Ripicephalus* ticks were observed during coat trimming.

Dermanyssus gallinae ticks were obtained from a layer farm diagnoses with ectoparasitism manifested by a lower egg production,

higher layer mortality and strong anaemia. The species of acari were determined by examination under the microscope and stereo magnifier.

Species of ectoparasitic insects

Haematopinus suis was harvested from a pig farm which displayed symptoms of ectoparasitism, manifested by scratching, anxiety and skin wounds. *Linognathus stenopsis* and *Damalinia caprae* were harvested from goats. *Linognathus vituli* and *Damalinia bovis* were harvested from a cattle farm. *Menopon gallinae* was harvested from layers reared in a household. *Goniodes meleagridis* was harvested from turkey hens reared in a household

Tested product

ROMPARASECT FORTE is a product manufactured by ROMVAC COMPANY S.A. It has the following composition

Cypermethrine .....2.5 g.
Diazinon .....12.5 g.
Excipient (dimethylformamidine, ethylic alcohol) ad .....100 ml.

Cypermethrine is a synthetic pyrethroid with strong action against parasites. It acts by contact and ingestion and it has neurotoxic effect inducing a state of hyper excitation followed by paralysis. Diazinon is a organophosphoric compound for synthesis, non systemic, who acts by inhibiting cholinesterase, accumulating acetylcholine which breaks down no more and which causes a block of muscular depolarization, the spastic paralysis of the ectoparasites.

ROMPARASECT FORTE was used in the experiment as watery emulsion in concentration of 2 ml/litre of water.

Testing protocol

The *in vitro* testing of ROMPARASECT FORTE was done on a number of 170 specimens of ectoparasites, of which 80 were acari species and 90 were insect species.

Twelve, 4.6 cm diameter, Petri plates were coated with round sheets of low porosity filter paper imbibed with 0.5 ml of the watery emulsion of ROMPARASECT FORTE. Each Petri plate was populated with 10 specimens of the following species: *Psoroptes cuniculi, Psoroptes bovis, Chorioptes bovis, Ripicephalus bursa, Dermanyssus gallinae, Haematopinus suis, Linognathus stenopsis, Linognathus vituli, Damalinia bovis, Damalinia caprae, Menopon gallinae and Goniodes meleagridis*.

The control specimens were assigned, 10 individuals each, in 5 Petri plates coated with round sheets of filter paper imbibed with 0.5 ml distilled water:

Control 1 – *Psoroptes cuniculi* Control 4 – *Hematophagous lice*
Control 2 – *Ripicephalus bursa* Control 5 – *Malophagous lice*
Control 3 – *Dermanyssus gallinae*

The results were interpreted by examination of the Petri plates under stereo magnifier at intervals of 2, 6, 12 and 24 hours after the parasites were put into contact with the tested product and with the distilled water; the motionless (dead) parasites were identified.

2. RESULTS AND DISCUSSION

Table 1 shows the effect of ROMPARASECT FORTE, watery emulsion in concentration of 2 ml/litre of water.

Table 1

***In vitro* effect of ROMPARASECT FORTE**

Ectoparasitic species	Number of specimens	Interpretation of results							
		2 hours		6 hours		12 hours		24 hours	
		Dead	%	dead	%	dead	%	Dead	%
Psoroptes cuniculi	10	8	80	2	20	-	-	-	-
Psoroptes bovis	10	10	100	-	-	-	-	-	-
Chorioptes bovis	10	9	90	1	10	-	-	-	-
Ripicephalus bursa	10	7	70	3	30	-	-	-	-
Dermanyssus gallinae	10	10	100	-	-	-	-	-	-
Haematopinus suis	10	10	100	-	-	-	-	-	-
Linognathus stenopsis	10	10	100	-	-	-	-	-	-
Linognathus vituli	10	10	100	-	-	-	-	-	-
Damalinia bovis	10	10	100	-	-	-	-	-	-
Damalinia caprae	10	10	100	-	-	-	-	-	-
Menopon gallinae	10	10	100	-	-	-	-	-	-
Goniodes meleagridis	10	10	100	-	-	-	-	-	-

Table 1 shows that after 2 hours of contact ROMPARASECT FORTE was 100% efficient against the acari *Psoroptes bovis,*

Dermanyssus gallinae and against the anoplura lice (*Haematopinus suis, Linognathus stenopsis, Linognathus vituli*), against the Malophagous lice (*Damalinia caprae, Damalinia bovis, Menopon gallinae, Goniodes meleagridis*), which means that all the specimens of ectoparasites were immobile (dead).

After 2 hours of contact the product was 70% efficient against the tick *Ripicephalus*, 80% against *Psoroptes cuniculi* and 90% against *Chorioptes bovis*. After 2 hours of contact the product ROMPARASECT FORTE was 100% efficient against all the species of ectoparasites under study.

Table 2

Viability of the negative controls in distilled water

Ectoparasitic species	Number of specimens	Interpretation of results							
		2 hours		6 hours		12 hours		24 hours	
		dead	%	dead	%	dead	%	Dead	%
Psoroptes cuniculi	10	-	-	-	-	-	-	2	20
Ripicephalus bursa	10	-	-	-	-	-	-	-	-
Dermanyssus gallinae	10	-	-	-	-	-	-	-	-
Hematophagous lice	10	-	-	-	-	1	10	1	10
Malophagous lice	10	-	-	-	-	-	-	-	-

Table 2 shows that no mortality was observed among the acari *Psoroptes cuniculi, Ripicephalus bursa* and *Dermanyssus gallinae* after 2, 6 and 12 hours of contact with distilled water. Ten percent of the hematophagous lice were dead after 12 hours of contact with distilled water and 20% after 24 hours. Twenty percent of the acari specimens *Psoroptes cuniculi* were dead after 24 hours of contact with distilled water.

3. CONCLUSIONS

3.1. ROMPARASECT FORTE watery emulsion in concentration of 2 ml/litre of water determined *in vitro* 100 % mortality after 2 hours of contact in the following species :

- *Psoroptes bovis ;*
- *Dermanyssus gallinae ;*

- *Haematopinus suis ;*
- *Linognathus vituli ;*
- *Linognathus stenopsis ;*
- *Damalinia bovis ;*
- *Damalinia caprae ,*
- *Menopon gallinae ;*
- *Goniodes meleagridis.*

3.2. The tested product induced 70 % - 90 % mortality after 2 hours of contact in the following species of ectoparasites:

- *Ripicephalus bursa* 70 %
- *Psoroptes cuniculi* 80 %;
- *Chorioptes bovis* 90 % ;

3.3. After 6 hours of contact with the tested product, 100% of the specimens of *Psoroptes cuniculi, Chorioptes bovis* and *Ripicephalus bursa* were dead.

3.4. ROMPARASECT FORTE, for external use, watery emulsion in concentration of 2 ml/litre of water, produced by ROMVAC COMPANY S.A. displayed an intense acaricide and insecticide action after a contact of at least 6 hours. Based on the results of the *in vitro* testing we recommend its use as disinfectant for the animal houses and paddocks, for equipment and tools.

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HEARTWORM SCREENING METHODS IN DOGS, CATS AND HUMAN

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Key words: heartworm disease, dirofilaria immitis, rapid immunochromatographic tests, heartworm screening methods, microfilariae

SUMMARY

Heartworm disease (a zoonosis disease which must be discovered and treated early enough in order to prevent its spreading) represents a real challange for veterinary practitioners becouse of its common clinical signs and most of all because of its cronical evolution. The fact that mosquito's bite is the most frequent cause of heartworm infection makes that the spreding area of the disease to be a large one (we can find dirofilariosa in every area where temperature and humidity are high) . The purpose of this paper is not only to raise awareness about the importance of heartworm diagnosis but to describe one of the most efficient diagnostic tool. Diagnostic methods should detect both microfilariae and antigen.

Dirofilariosis is a zoonosis described in domestic and wild animals like dogs, cats, wolves and foxes. *Dirofilaria immitis (fig.1)* and *Dirofilaria repens* are the two most common filarial nematode worms responsable with producing the infection. The most frequent route of infection spreading is by female mosquito's bite (the male is not able to develop the life cycle of *Dirofilaria immitis*). Important to know is that hemotransfusions can also cause the desease transmision if the donor's blood is contaminated. Dirofilariosis can be prevented with profilactic medication. On the other hand, the absence of a post–infection treatment or innadequate drug administration can lead to death. In the last few years, concurrent with international trade development, the incidence of dirofilariosis in Romania has increased. The intermediate host of *Dirofilaria Immitis* (the mosquito) finds in Romania proper development conditions: temperatures of over 14 degrees and high humidity.

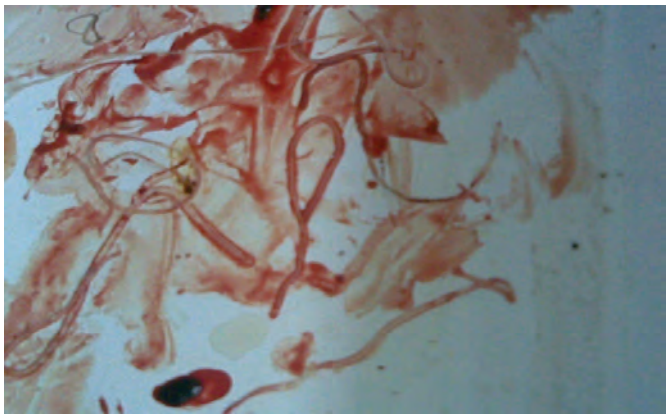

Fig1. Adult heartworm in Infectious Diseases Department.Veterinary Faculty Bucharest- original

In most of the human cases of dirofilariosis, *Dirofilaria repens* is the etiological agent that causes cutaneous changes. Literature does not mention any case of human dirofilariosis in our country, as opposed to Hungary, where the first case is mentioned in 1879. It seems that the number of cases gradually increased lately if we consult the latest research. The diagnosis in these cases was made following a detailed case history and after a careful examination of the parasites found in different areas: subconjunctival space and the subcutaneous tissue of the thigh, forearm.[3]

The most important elements that contribute to the increasing cases of dirofilariosis in Romania consist of: the climatic conditions, international trade development and the fact that people travel along with their pets, bringing in contaminated animals from countries with a high dirofilariosis prevalence. There is little information available about the incidence of dirofilariosis in our country. However, infections in dogs ranges from 2-17% in Bulgaria, Greece and Turkey up to 65% in Romania and some areas considered to be endemic[4]. Spiru Haret University published some studies about the heartworm incidence in Romania: 12 out of 52 examined dogs were detected as beeing contaminated[4]. The lack of information has led to the creation of National Epidemiological Surveillance Network of Infectious and Parasitic Disease in Pets – **PetEpiNetVet -,** a network that provides assistance to owners and veterinarians.

The clinical signs that follow the heartworm infection consist of: anorexia, ascites, edema, tachycardia, tachypnea, cahexie, weight loss, syncope, cough, cough with blood streak, epistaxis, paresis - paralysis, cutaneous nodules, right heart failure - in case of massive contamination

with filaria- , flu pipe. All these clinical signs should lead us to a confirmation diagnosis that can be done by at least two ways.

Heartworm treatement includes two aspects: heartworm prophylaxis that consists of drugs which belonging to macrocyclic lactones or macrolides class (ivermectin, selamectin, moxidectin) and adulticide therapy (milbemycin, ivermectin)[2].

If any specific signs are found during clinical examination, the animal needs further testing in order to see if a heartworm infection occured**.** If the test is positive there is no need for another diagnosis tool. On the other hand, if the test is negative but the animal continues to present specific simptoms an immunologic diagnosis is necessary. Microfilariae detection can be done by: fresh blood examination, concentration methods (Knott test and the filter test) and histochemical exam. *Dirofilaria immitis* microfilariae indentification based on morphology is considered a definitive proof of infection (specificity 100 %).

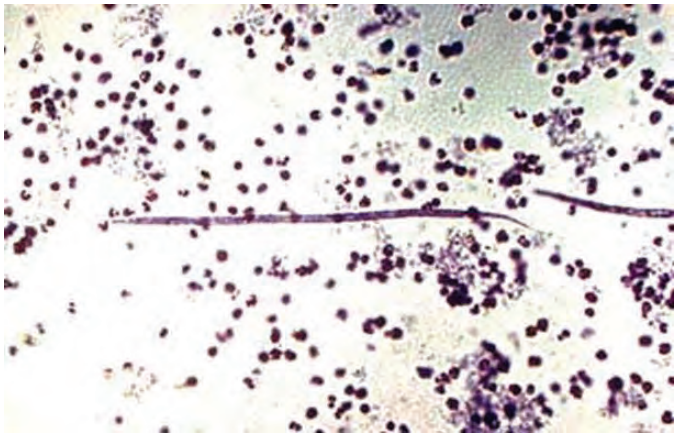

Fig.2 Microfilariae of *Dirofilaria Immitis*

For many years, the modified Knott test was used as a surveillance method[5,6]. Yet it turned out to be ineffective, being unable to detect sterile and unisexual infections. Taking that into consideration, nowadays rapid tests and serological tests[7] (for specific FILARCHEK antibodies or antigens) are preffered. Recent research show that antigen detection tests are more reliable than the ones that detect microfilariae. For detection of heartworm specific circulating antigen, serological tests and immunochromatographic rapid tests are used. Although the two methods present a high sensitivity (the difference is insignificant), there are very few cases when they give false negative results.[8]

There are some of the serological tests used for heartworm surveillance/diagnostic:

ELISA

1. detecting adult forms antibodies (AB-ELISA);
2. detecting adult forms antigens (AG-ELISA);

IMMUNOFLUORESCENCE

1. detecting microfilariae - specific antibodies (MF-IFA);
2. detecting specific adult forms antigens (AG-IFA);[8]

The efficacity of HEARTWORM IC – BIOPRONIX – AGROLABO was demonstrated by Agrolabo specialists in several clinical studies. Also, our unpublished studies revealed their practical value and that they are a very helpful diagnosis tool for field veterinarians. It is a simple and fast method; the results can be interpretated in 10-15 minutes; it doesn't require special equipment and can-be made at any veterinary clinic or field;it has a sensitivity of 97.98%.

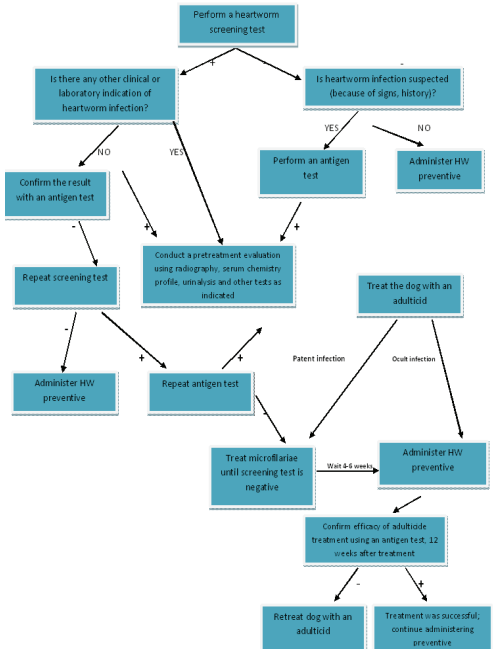

Fig.4 Recommended protocol for diagnosing and treating heartworm disease (Charles Courtney - University of Florida)

TEST PRINCIPLE

Heartworm IC is a rapid test that employs the immunochromatographic sandwich technique using monoclonal antibodies against *Dirofilaria Immitis* antigen. A monoclonal antibody conjugated with colloidal gold and a further monoclonal antibody are immobilised on the test membrane. Samples containing *Dirofilaria immitis* antigen will bind to the colloidal gold conjugated antibody forming an antigen-antibody complex. The complex migrates along the membrane and is then captured by the second monoclonal antibody that is immobilised on the membrane at the level of window number two, forming a red line. Other capturing antibodies are immobilised at window three. These will bind with the fluid that continues to migrate along the membrane, forming a second red line which indicates that the test has been carried out correctly. This line should appear whether the test result is negative or positive.

TEST PROCEDURE

1. Using the appropriate pipette, dispense one drop of whole blood, serum or plasma sample into window no. one of the device.
2. Add two drops of diluent to window no. one of the device.
3. After 10 minutes read the result, do not read the result after more than 15 minutes
4. Result interpretation.

RESULT INTERPRETATION

If the test is negative, a red line will only appear in window number (internal control line).

If the test is positive, two red lines will appear: one in window number two (test line) and one in window number three (internal control line).

The test is considered invalid if no line appears in window number 3 of the device (internal control line), even if a line appears in window number two (test line). (fig. 3)

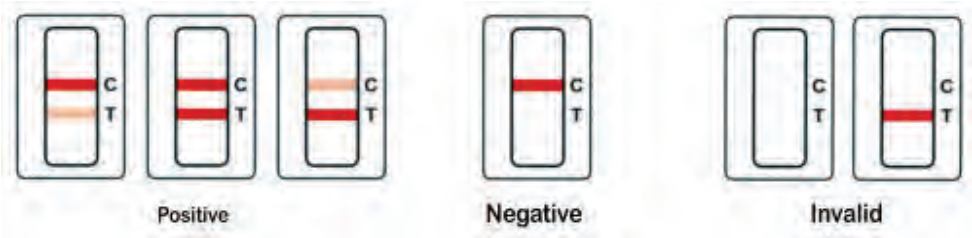

Fig 3. Result interpretation

For a complet screening veterinarians should use two diagnostic methods: one for microfilarae and the other for specific antigen detection. Moreover, for an efficient diagnosis it is important to choose high sensitivity and specificity tests.

Important to note is that currently, the easiest and most suitable method for screening is the immunochromatographic test - it is fast (results in 10 minutes) and easy to use.

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THE PREVALENCE OF *TOXOPLASMA GONDII* INFECTION IN CATS FROM HUNEDOARA COUNTY

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Key words: prevalence, cats, Arad, *Toxoplasma gondii*

SUMMARY

To establish the prevalence of Toxoplasma gondii infection in cats from Hunedoara county, 42 samples of serum and faeces were tested serological and coproscopical.
From all samples, 59.52% were positive and 40.48% were negative. The seroprevalence was higher in household European breed adult male cats from rural area fed with raw meat.
Coproscopically, we didn't detect T. gondii oocysts in any sample.

Toxoplasmosis is a protozoosis produced by the coccidian of *Toxoplasmidae* family. Over 350 vertebrate species are being parasitized at internal organs and tissue levels, this parasite being the most spread protozoa from this point of view. The parasite can be meet all over the world fact that proves its medical and veterinary medical importance (Dărăbuş *et. al*., 2006). Due to its zoonotical importance, *T. gondii*, is the most studied coccidian.

To the final host – a feline – evolves asymptomatic. The economical losses are important due to important low birth-rate and because of abortion to the important livestock, especially to the sheep and swine. Man is receptive to *T. gondii*, and represents the final host for this parasite (Dărăbuş *et*. al., 2006).

Internationally signalization of toxoplasmosis increased incidence to human being and animals, and the reduced number of the bibliographical data from our country regarding the toxoplasmosis infection to animal and human being justify the purpose of our research.

1. MATERIAL AND METHOD

During July 2008 – January 2009 there were collected 42 samples from Hunedoara County.

In order to do some association as far as it regards the prevalence of the infestation with *Toxoplasma gondii* to cats, we followed some receptivity factors that we could associate with toxoplasmosis.

Faeces and serological samples came from cats with the age ranged between 4 months and 9 years. A number of 16 cats with ages between 4 months and one year, representing the younger animals, and 26 adult cats were over 2 years.

Out of the total cats studied, 21 were males and 21 were females.

As regarding the breeds, 23 were common European cats, 10 Burma cats, 5 Persians and 4 Siamese.

Cats were both from urban and rural area.

From the 20 urban cats, 8 of them were kept in apartment and never were outside, and the other 12 cats were also kept inside the house but they had free access outdoor. The 22 cats that came from rural area were animals kept near the house along with other animals (sheep, swine, horses, poultry and dogs).

Data regarding the crude meat consumption were different. Of 42 studied cats, 32 cats ate at least once in their life crude meat; most of them had the possibility to hunt, as they were living in houses with yards and gardens. Instead 10 of the cats owners declared they never fed cats with crude meat.

a). The coproscopic examination

The microscopically examination of the preparation made up by final hosts' faeces (cats), was carried out at the Parasitology and Parasitical diseases department of the Veterinary Medicine Timisoara, Romania, by floatation with a sugar solution and Willis.

b). ELISA method

The collected blood was left to decantation in order to examine the serum, and the serum was kept into the freezer till January 2009, when samples were processed at the Parasitological and Parasitical diseases of the Veterinary Faculty department, Timisoara

Serum samples were indirectly examined by ELISA method, using ID-VET Screen Multi-species kits to emphasise the distinctive anti toxoplasmosis Ig G antibodies, obtained as a result of a *Toxoplasma gondii* infestation. The ELISA technique is describe in the kit's papers.

Optic densities achieved after reading the plate were explained after the following calculation:

$$\text{Titrul Atc.} = \frac{\text{DO probă}}{\text{DO pc}} \text{ X } 100$$

Atc. – antibodies (Probă) sample – examined sample
DO – optical density PC – positive control

Values over 200% were considered heavily positive, values between 50 and 200% were positive, the ones between 40 and 50% were uncertain and the values under 40% were considered negative.

2. RESULTS AND DISCUSSIONS

a). The coproscopic examination

After faeces examination by floatation method with sugar solution and Willis, we could not identify oocysts of *Toxoplasma gondii* or *Isospora* type. These result it can be explained by the fact that cats discharge only few days on their life oocysts (10 to 20 days), and re-infestations with oocysts discharge are extremely rare.

Some samples exhibited though other parasitized species. Of 42 examined samples only 4 were identified as having *Toxocara* spp.

38 samples were negative copro-parasitological speaking.

b). The ELISA technique

From 42 examined samples in Hunedoara County, 25 were positive to the toxoplasmosis infestation (59.52%), and 17 were negative (40.48%). The 25 positive samples were associated with different receptivity agents, as it follows:

- cats had an age ranging from 1 to 9 years;
- 7 cats were Burmese breed, 4 were Persian breed and 14 were European common cat;
- 11 cats were females and 14 were males;
- 16 were from rural area and 9 from urban area;
- 18 cats had free access to outside area and 7 cats were permanently kept indoor;
- 18 have eaten crude meat and 7 cats never ate crude meat.

In order to analyse the involvement of some receptivity agents in toxoplasmosis prevalence for the studied county, we considered the following factors: age, breed, gender, area where they came, life environment and alimentation type of the cat.

We achieved varied results by correlating the studied factors regarding the positive data about *Toxoplasma gondii* infestation.

To the young category, with ages between 4 months to 1 year (n=15), only three samples were positive ((20%), the exact ages were 8 months, 10 months and 1 year, respectively. The other 12 samples were negative (80%).

Out of 27 adult cats, with ages between 1.2 years to 9 years, 22 were positive (81/48%), iar 5 samples were identified as negative (18.52%) (fig.1).

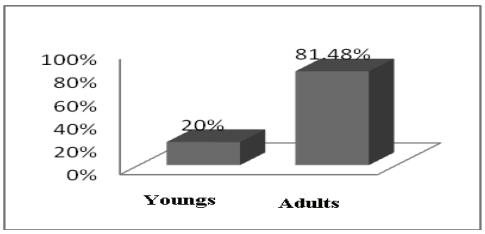

Fig. 1 Graphical presentation according to the age

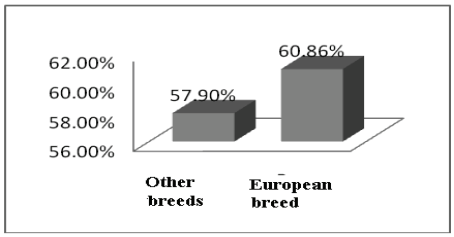

Fig. 2 Graphical presentation of the positivity according to the breed

From the total of 23 examined samples, taken from European common cat, 14 samples were positive (60.86%) at the ELISA test, and 9 samples were negative (39.14%).

The other 19 individuals were from different breeds: 10 Burmese, 5 Persians, and 4 Siamese. There were 11 samples positive to the *Toxoplasma gondii* infestation (57.9%) (fig 2).

As it regards the gender, the results were: 21 females and 21 males. From males, 14 cats exhibited anti-*Toxoplasma* antibodies (66.66%), and 7 did not show any sign (33,33%). From the females 11 were positives (52,38%) in *Toxoplasma* infection and 10 were negatives (47,62%) (fig 3).

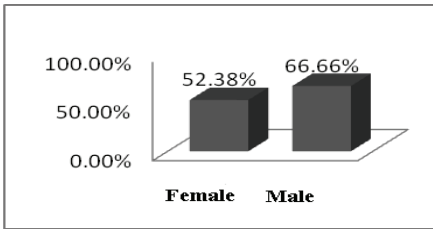

Fig. 3 Graphical presentation according to the sex

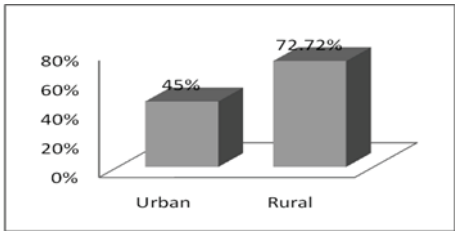

Fig. 4 Graphical presentation according to the area

As it regards the area where the cats came from, there were collected 20 samples from urban area cats and 22 from rural are cats.

For the urban area cats 9 samples were identified as positive (45%), and 11 were identified negative (55%). In the rural area, 16 samples were found as positive to the *T.gondii* (72.72%), and 6 samples were negative (27.28%) (fig. 4).

We also considered as a criteria that could influence the prevalence of the infestation with *T.gondii*, the life environment of the cat: house or apartment. 34 cats had free access outdoor; they live in farms or countryside houses. On these conditions, we identified 18 positive samples (52.94%), and 16 negative samples (47/06%).

Of the 8 cats which lived within a flat, there were found positive 7 cats (87.5%) and only one cat (12.5%) was identified negative to the Toxoplasma gondii infestation (fig. 5).

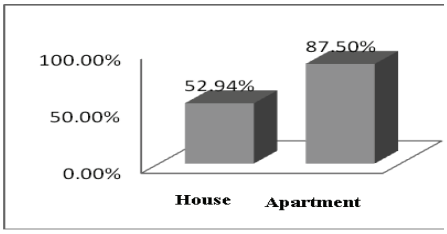

Fig. 5 Graphical presentation according to the life environment

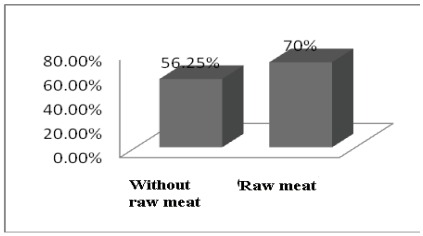

Fig. 6 Graphical presentation according to the alimentaion

The last factor studied and considered in the same time one of the main factors involved on disease transmission was the cats' diet, namely if the cats receive crude meat or if they have the possibility to hunt. On this class were 32 cats that have eaten meat at least one time and 10 cats that never ate crude meat. From the total of 32 cats, 18 cats were diagnosed as positive (56.25%), to the toxoplasmosis infestation and 14 had been diagnosed negative (43.75%). For the cats that are supposed they never ate crude meat, 7 individuals were identified as positive (70%), and 3 were negative (30%) (fig. 6).

Data we achieved during this study are in accordance with the existent literature. From the attained percentage during our experiment we observed the influence of different factors on the prevalence of the disease. It was observed that *Toxoplasma gondii* is more frequent to the adult cats and has a lower impact to the young cats that does not hunt.

This disease is frequently met in the rural area linked also with the environment agents, namely cats that have access to the outside and get in contact with other cats and animals, and is also linked with the crude meat factor from cat feed, gave by the owner or hunted. This last factor supports the major toxoplasmosis infestation route of the cat, with tissue cysts filled with bradizoids from the infested meat (table 1).

Table 1.

The principal factors asociated with *Toxoplasma gondii* infection

Indicator	Positive samples/total samples (%)
Young	3/15 **(20%)**
Adult	22/27 **(81,48%)**
Other breeds	11/19 **(57,90%)**
European breed	14/23 **(60,86%)**
Female	11/21 **(52,38%)**
Male	14/21 **(66,66%)**
Urban	9/20 **(45%)**
Rural	16/22 **(72,72%)**
House	18/34 **(52,94%)**
Apartment	7/8 **(87,5%)**
Raw meat	18/32 **(56,25%)**
Without raw meat	7/10 **(70%)**

Regarding the gender the percentage differences are not quite high, still males are more frequent infested because they hunt more often than females and they have a better resistance to the disease than the females. This statement is not generally available because some studies, there were achieved reverse results (Titilincu, 2008).

Breed factor doesn't really influence the prevalence of the disease. Although for the breed factor, to the urban area and for the life environment the percentage of positive samples was high, considering that these values are not to relevant because of the small number of examined samples, subsequent studies being necessary.

The 4 positive cats to the coproscopic examination presented antibodies Ig G anti-toxoplasmosis. These cats lived in rural area, with ages from 3 to 9 years, and tree of them were common European and one Burmese. They have the possibility to hunt.

There were bizarre cases where cats never went outside and never have eaten raw meat, but they had anti-*Toxoplasma* Ig G antibodies. This fact proves that in addition to the factors mentioned above there are other factors, considered less important, that influence also the contamination. These could be: oocysts air circulation, brought in house on the shoes and even by the cockroaches. It should be good to mention that even tap water, non boiled milk, or the grass we bring in for cat to eat can represent contamination factors.

Along these cases we found some interesting cases. Some adult cats adopted from the street, did not had anti- *Toxoplasma* specific antibodies. Or cats that lived together in the same environment, ones

had antibodies others didn't. This fact emphasises the importance of the resistance, feeding behaviour and hygiene of each individual.

For the county were we carried out our research, the information are the more important as they are the first data reported regarding the infestation with toxoplasmosis.

Worldwide there are very diverse results. The seropositivity degree (ELISA) increases on the same time with the age: 22% of the cats under a year and 80% for the ones over 10 years. And between 9 to 46% of the cats kept as pet from Europe and USA presents to the serological test exposed in the past to the past, while sero-prevalence of the toxoplasmosis in Asia was estimated between 6 to 9% (Dubey, 2005). In Mexico, the positive infection was about 21.8% (Besne-Merida, 2008). In Brazil, 25% of the cats had contact with the parasite, a higher prevalence being encountered to the cats with ages higher than one year (Bresciani, 2006 In Portugal 3.9% of the tested cats had the antibodies titre of 20; 23.7% had a titre of 40, and 72,4% presented a titre higher than 800 (Lopes, 2008). In Belgium, 2% of the cats with ages under 12 months presented anti-toxoplasmosis antibodies, and 44% of the cats were positive around the age of 7 years (De Craeye, 2008).

The infestation of the cats from the studied county is important because of the disease transmission possibility to the livestock and to people. Thus, it is necessary the implementation of some supervising programs for the disease in order to reduce the environment infestation degree and subsequent of the economical losses produced by abortions as well to people especially pregnant women and imunosupressed persons.

3. CONCLUSIONS

3.1. In the Hunedoara County, the prevalence of the toxoplasmosis in cats was about 59.52%.

3.2. The seroprevalence of *Toxoplasma gondii* in cats is influenced by age, gender, breed, provenance area, are where are kept (outdoor or indoor), and feeding manner of the cats.

3.3. There were identified parasitary infestation with *Toxocara spp*.

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TRACE ELEMENTS IN ORGANS AND TISSUES OF DOLPHINS STRENDED ON THE BLACK SEA COAST

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Key words : heavy metals, intoxication, residual, trace elements

SUMMARY

The distribution of metals (Pb, Cd, Cu and Zn) were investigated in various tissues and organs obtained from dolphins stranded along the Black Sea Coast from Romanian during 2007-2009, founded by prowl car of the "Oceanic Club" NGO Constanta.

Metals were determined by atomic absorption spectrophotometry inside the ***Zonal*** (Regional) Veterinary ***Laboratory*** for ***Residue*** Control Constanța. Lead concentrations were generally high in the flipper, intervertebral disc, testicle, ovary, intestine, stomach, spleen, liver, brain, bone and low in melon, lung, tongue, heart, kidney, bubbler . Some metals showed organ-specific accumulations: copper and zinc exhibited high concentrations in liver, the highest cadmium concentration was observed in liver, kidney, intervertebral disc, flipper. Pathological, microbiological and parasitological surveys were performed on this dolphins. It was not possible to relate dolphin death to a specific cause, or to contaminants; however, the accumulation of metals may contribute to certain pathological alterations and represents a risk factor for dolphins. The main couse of stranded dolphins represented their catching with fishing gears (gill nets).

INTRODUCTION

Three dolphin species can be found in Romanian Black Sea waters: the harbour porpoise (*Phocoena phocoena*, 400-600 individuals), the bottle-nosed dolphin (*Tursiops truncatus*, 500-1000 individuals) and the common dolphin (*Delphinus delphis*, 600-800 individuals).

(***http://www.delfini.ro/marea-neagra.php).

Massive pollution by heavy metals, was reported several years ago and it sharply increased after the war in Yugoslavia, especially following the bombing of chemical plants located on the Danube in Yugoslavia.

Marine mammals are top predators are the trophic pyramids, are indicators of the health of marine ecosystems. Thus, dolphins concentrate heavy metals (Cu, Zn, Co, Ni, Cd, Cr, Pb, Mn) and radioisotopes (137Cs) (Crathorne, B., AJ Dobbs, and Y. Reese, 1996).

The metal contamination depends on certain individual characteristics - physical, physiological, age, sex. In general, the malnourished animals implicate greater sensitivity , due to a reduction in fat storage opportunities and the effect of the deposit in demanding structures (liver, kidney) (Aguilar, A., A. Boreel, and T. Pastor,1999).

Populations of three species of dolphin living in the Black Sea are currently in critical condition. Even if the capture of dolphins has been banned in Romania in 1966 their population numbers have steadily declined, as a result of pollution.

MATERIAL AND METHOD

The distribution of metals (Pb, Cd, Cu and Zn) were investigated in various tissues and organs obtained from dolphins stranded along the Black Sea Coast from Romanian during 2007-2009, founded by prowl car of "Oceanic Club" NGO Constanta. The concentrations of metals from analysed samples are reported in Tables 1, 2, 3, 4, 5, 6, 7.

Metals were determined by atomic absorption spectrophotometry inside the ***Zonal*** (Regional) Veterinary ***Laboratory*** for ***Residue*** Control Constanta. Method used - SR EN 14082: 2003 Foodstuffs - Determination of lead, cadmium, zinc, copper and iron by atomic absorption spectrometry (AAS) after calcination.

Equipment used: flame atomic absorption spectrophotometer GBC Avanta PM.

This method determinated the concentration of a chemical element in the sample research subject, by measuring the absorption of electromagnetic radiation of a specific wavelength of its passage through the environment containing steam as uniformly distributed, free atoms of elements that are investigating. (Popescu, N . et al., 1986) .

Method is based on samples preparation according to EN 13804: 2002:

- Samples are minced and homogenized, then in a capsule or porcelain crucible are weigh 10-20 grame of tissue, are inserted into the electric furnace, with temperature programmed at 450 ° C with gradual increase temperature for calcinations.

-The obteined ash is dissolved with hydrochloric acid(SR EN 14082:2003) and then the obtainted residue is evaporated to dryness. After that the residue is redissolved whit 0.1 M nitric acid solution and pass quantitatively to volumetric flask.

Do to the sample, is preparated a blank of reagents used for mineralization. From mineralized sample is determined the lead,

cadmium, copper and zinc with Flame atomic absorption spectrophotometer. For reading the samples is making the calibration curve.

The calibration curve is determined by preparing standard solutions for the elements that are used to this research

Results expression:Formula

E(mg / kg) = (C sample - C blank) x V x dilution / m, in which:

E = element name,

C = matal concentration reading on calibration curve (mg/ml)

V = total volume of sample solution (50 or 100 ml),

m = quantity of sample taken into work, in grams,

2. RESULTS AND DISCUSSION

Table no.1.

Case no 1. Dolphin *Tursiops Truncatus ponticus* stranded in 23.10.2007

No.	Tissues	Lead mg/Kg	Cadmium mg/Kg	Copper mg/Kg	Zinc mg/Kg
1.	Stomach	0,74	0,175	0,88	15,98
2.	Ganglions 1	0,55	0,164	0,92	17,89
3.	Ganglions 2	0,89	0,153	0,85	17,60
4.	Heart 1	0,28	0,068	3,74	25,00
5.	Heart 2	0,20	0,061	3,46	18,30
6.	Tongue	Ned.	0,050	0,65	18,20
7.	Testicle	0,72	0,115	0,90	29,30
8.	Flipper	1,77	0,342	1,52	60,30
9.	Intestine	0,70	0,107	0,94	19,90
10.	Spleen	0,67	0,135	0,99	26,90
11.	Kidney	0,26	0,187	2,09	21,10
12.	Lung	Ned.	0,073	0,83	25,50
13.	Muscle 1	Ned.	0,078	1,22	17,80
14.	Muscle 2	Ned.	0,067	1,30	16,80
15.	Muscle 3	0,72	0,151	1,29	38,10
16.	Liver	0,24	0,094	7,99	43,10

1.Highest lead concentration was detected in the flipper, having a concentration of 1.77 mg/kg and then decreasing in lymph, muscle, testicle, spleen, heart, kidney and liver.

2.Highest cadmium concentration was detected in the flipper, 0.342 mg/kg, then decreasing in kidney, stomach, lymph nodes, muscle and spleen .

3.Highest copper concentration was detected in liver (7.99 mg/kg), and then descreasing in heart, kidney and flipper.

4.Highest zinc concentration was detected in the flipper (60.30 mg/kg), then descreasing in liver, muscle, spleen, kidney, lung, heart and testicle.

Table no.2.

Case no.2. Dolphin *Tursiops truncatus ponticus* –famele,stranded in 29.04.2008

No.	Tissues	Lead mg/Kg	Cadmium mg/Kg	Copper mg/Kg	Zinc mg/Kg
1.	Flipper	0,23	ned	0,42	11,63
2.	Brain	0,08	0,023	2,01	13,99
3.	Ribs	0,33	0,019	0,35	59,27
4.	Intestinal content with fish	0,14	0,038	1,76	23,30
5.	Heart	ned	0,016	2,80	30,55
6.	Intervertebral disc	1,48	0,140	1,32	149,85
7.	Liver	0,03	0,069	19,66	33,08
8.	Tongue	0,07	0,017	0,89	16,99
9.	Ovary	Ned	0,039	1,39	12,79
10.	Lung	0,05	0,018	0,93	24,91
11.	Kidney	Ned	0,081	2,71	18,92
12.	Muscle 1	Ned	0,012	1,62	21,39
13.	Muscle 2	Ned	0,016	1,17	17,03
14.	Blubber	0,26	0,005	0,39	3,12

1. Highest lead concentration was detected in the intervertebral disc, with a concentration of 1.48 mg/kg and then decreasing in the ribs, flipper, blubber, intestinal content with fish.

2. Highest cadmium concentration was detected in the disc intervertebral, 0.140 mg/kg, then decreasing in kidney, liver, intestinal contents of fish and ovary.

3. Highest copper concentration was detected in the liver (19.66 mg/kg), then decreasing in the kidney, brain, muscle tissue, the intestinal content of fish, ovary and disk.

4. Highest zinc concentration was detected in the intervertebral disc (149.85 mg/kg), then decreasing in ribs, liver, heart, lung, kidney, muscle tissue and gut contents of fish.

Table no.3.

Case no.3. Dolphin *Tursiops truncatus ponticus*-male-stranded in 5.05.2008

No.	Tissues	Lead mg/Kg	Cadmium mg/Kg	Copper mg/Kg	Zinc mg/Kg
1	Brain	0,49	0,016	2,23	15,46
2	Heart	0,04	0,007	2,18	39,16
3	Liver	0,17	0,020	25,37	43,43
4	Lung	0,25	0,005	0,87	34,02
5	kidney	ned	0,027	3,35	22,84
6	Muscle	0,12	0,014	1,61	25,69
7	Testicle	0,03	0,013	0,80	21,89
8	Blubber	0,16	0,011	1,43	20,47

1.Highest concentration of lead was detected in the brain (0.49mg/kg) and then decreasing concentrations in lung, liver, fat and muscle tissue.

2.Highest concentration of cadmium was detected in kidney (0.027mg/kg), then decreasig in liver, brain, muscle tissue, testicle and fat.

3.Highest concentration of copper was detected in liver (25.37mg/kg), then decreasing in the kidney, brain, heart, muscle tissue, fat and testicle.

4. Highest zinc concentration was detected in the liver (43.43mg/kg), then decreasing in heart, lung, muscle tissue, kidney and testicle.

Table no.4.

Case no.4. Dolphin*Tursiops truncatus ponticus*-sex unknown-alterated

No.	Tissue	Lead mg/Kg	Cadmium mg/Kg	Copper mg/Kg	Zinc mg/Kg
1	Fragment of bone	0,20	0,011	0,30	40,32

Table no.5.

Case no. 5. Dolphin *Phocena Phocena*-male-standed in 27.06.2008

No.	Tissues	Lead mg/Kg	Cadmium mg/Kg	Copper mg/Kg	Zinc mg/Kg
1	Liver	ned	ned	1,61	16,72
2	Lung	ned	0,013	1,37	20,06
3	Kidney	ned	0,244	2,13	14,60
4	Muscle	ned	ned	1,61	16,72

1.In liver tissue, kidney, muscle and lung samples were not detectable residues of lead.

2.Highest cadmium concentration was detected in the kidney (0.224 mg/kg.)

3.Highest copper concentration was detected in the liver (2.13 mg/kg), then decreasing concentrations in other tissues.

4. Highest zinc concentration was detected in the lung (20.06 mg/kg), then decreasing in liver, muscle and kidney tissues.

Table no.6.

Case no. 6. Dolphin *Delphinus Delphis* stranded in 20.07.2008

No.	Tissues	Lead mg/Kg	Cadmium mg/Kg	Copper mg/Kg	Zinc mg/Kg
1	Liver	0,33	0,170	1,99	53,07
2	Muscle	0,24	0,065	0,79	13,67

1.Highest concentration of lead, cadmium, copper and zinc was found in the liver.

Table no.7.

Case no.7. Dolphin *Tursiops truncatus ponticus*-Famele stranded in 14.04.2009

No.	Tissues	Lead mg/Kg	Cadmium mg/Kg
1.	Tongue	ned	Ned
2.	Kidney	ned	0,049
3.	Heart	ned	Ned
4.	Cartilage	0,10	0,013
5.	Liver	ned	0,051
6.	Muscle	ned	Ned
7.	Bone	0,11	0,011
8.	Stomach content	ned	0,034
9.	Brain	ned	Ned
10.	Melom	0,22	Ned
11.	Ovary	1,20	Ned

1. Highest lead concentration was detected in the ovary (1,20 mg/Kg), and then decreasing concentrations in melom, bone and cartilage.

2. Highest cadmium concentration was detected in the liver, (0,051 mg/kg), then decreasing in kidney, stomach content, cartilage and bone.

3.CONCLUSIONS

3.1.Lead concentrations were generally high in the flipper, intervertebral disc, testicle, ovary, intestine, stomach, spleen, liver, brain, bone and low in the melon, lung, tongue, heart, kidney and bubbler .

3.2 Copper and zinc was detected in all tissues and high concentrations was founded in the liver.

3.3.The highest cadmium concentration was observed in the liver, kidney, intervertebral disc and flipper.

3.4.The accumulation of metals may contribute to certain pathological alterations and represents a risk factor for dolphins.

THANKS

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CONTAMINATION WITH POLYCHLORINATED BIPHENYLS (PCBS) AND ORGANOCHLORINE PESTICIDES OF DOLPHINS STRENDED ON THE BLACK SEA COAST

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Key words : organochlorine, polychlorinated biphenyl, dolphin, Black Sea

SUMMARY

This study realives the contamination with polychlorinated biphenyl and organochlorine pesticides in bubler samples from dolphins strended on the Black Sea Coast (*Tursiops truncates ponticus, Phocena phocena* and *Delphinus delphis*), founded between 2007 to 2009, by prowl car of the "Oceanic Club" N.G.O Constanta. Residues are determined by gas chromatography with electron capture detection, inside the ***Zonal*** (Regional) Veterinary ***Laboratory*** for ***Residue*** Control Constanța. Polychlorinated biphenyls and organochorine pesticides were determinated in bubbler samples from 18 dolphins strended. The highst concentration of organochlorine pestiocides was registreted at lindane (4,320 mg/Kg lipid weight), beta HCH (12,151 mg.Kg lipid weight) and DDTs (47,260 mg/Kg lipid weight). PCBs concentrations ranged from 0,818 to 9,504 mg/Kg lipid weight.

INTRODUCTION

On a global level, human rely on marine resources for a wich range of activities, for recreational,aesthetic and economic reason (Himes, 2003).

Just in recent times people started to become aware that the oceans do not contain an endless source that they can not eternally dilute pollution or absorb the impact of shore development (Granek et al., 2005).

Ruckelshaus and Hays (1998) point that the primary sources of biodiversity decline in the marine environment are: overfishing, pollution, habitat distruction and fragmentation, introduction of nonindigenous species and climate change.

The impact of pollution on marine wildlife is broadly recognised (Ruckelshaus & Hays, 1998; Tuerk et al., 2005). Organochlorine compounds used in agriculture can reach coastal and estuarine areas (Crespo & Hall, 2001).

These substances and heavy metals from industries can be transferred to marine mammals mainly through their diet (Biorge, 2001).

This substances tend to be acumulated in the fat tissues and concentrated across trophic levels, with can increased poisonous effect from preys to top predator.Marine traffic is not only a major cause of pollution but it generates disturbance and noise which may impact cetacean population (Notarbartolo di Sciara, 2003). Seismic oil exploration is another sourse of noise pollution.

1.MATERIAL AND METHOD

This study realives the contamination with polychlorinated biphenyl and organochlorine pesticides in bubler samples from dolphins strended on the Black Sea Coast (*Tursiops truncates ponticus*, *Phocena phocena* and *Delphinus delphis*), founded between 2007 to 2009, by a prowl car of the "Oceanic Club" N.G.O Constanta. Residues are determined by gas chromatography with electron capture detection, inside the ***Zonal*** (Regional) Veterinary ***Laboratory*** for ***Residue*** Control Constanța. Polychlorinated biphenyls and organochorine pesticides were determinated in bubbler samples from 18 dolphins strended.

The method used the determination of polychlorinated biphenyl (PCBs) and organochlorine pesticides in food of animal origin by gas chromatography in conformited :

- SR EN 1528: 1-4:2004 - Food fat. Determination of polychlorinated biphenyl (PCBs) and organochlorine pesticides.
- AOAC 970.52 – Organochlorine and organophosphorus pesticide residues. General multiresidues method (Year 1997 ed.16 rev3).
- Equipment used: Gaz cromatograph type Varian – used for separation, identification and quantification of organochlorine pesticides residues present in samples.
- Substances used for making curve of calibration:

- α HCH (α-BHC) (α-1,2,3,4,5,6-hexachlorocyclohexane) ;
- β HCH (β-BHC) (β-1,2,3,4,5,6-hexachlorocyclohexane) ;
- γ HCH (γ-BHC ; lindane) (γ -1,2,3,4,5,6-hexachlorocyclohexane) ;
- o,p'-DDE (o,p'- [1,1-dichloro-2,2-bis (4-chlorophenyl) ethylene)] ;
- p,p'-DDE (p,p'- [1,1-dichloro-2,2-bis (4-chlorophenyl) ethylene)] ;
- o,p'-DDD [o,p'-1,1-dichloro-2,2-bis (4-chlorophenyl) ethane] ;

- o,p'-DDT [o,p'- (1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane] ;
- p,p'-DDD [p,p'- 1,1-dichloro-2,2-bis (4-chlorophenyl) ethane] ;
- p,p'-DDT [p,p'- (1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane] ;
- hexaclorbenzen, heptaclor si heptaclor-epoxid, aldrin, dieldrin, endrin, PCB 28, 52, 101, 153, 138, 180, 118.

Method principle: Extraction of the residues, together with the fat, from the sample matrix by the use of pethroleum ether. Removal of the solvent by evaporation and determination of the fat content by weighing out the mass of the remainder. Redissolving the extract in pethroleum ether and adding the internal standard. Chromatography using a florisil column, with mixture of pethroleum and ethyl ethers as the eluting solvents. Residues in concentrated eluates are measured by GC and identified by gas chromatography.

Method steps:

-Thoroughly mixed sample is extracted with petroleum ether.

-3g of the fat is weighted and partitioned between petroleum ether and acetonitrile (ACN).

-Entire solution of acetonitrile (ACN) is diluted with water and residues are extracted into petroleum ether.

-Residues are purified by chromatography on Florisil column, eluting with mixture of petroleum and ethyl ethers.

-Concentration of eluates by rotavapory concentrator

-Identification and determination - purified extracts are analysed by GC with electron capture detection (ECD).

The identification of peaks is made by coincidence of retention time and a given reference window.

Determination of peaks calibration system based on a multilevel calibration curve (6 points), linear, point to point force, zero included comparison between sample and calibrant by area, with manual adjustements if necessary.

Concentration of polychlorinated biphenyl and organochlorine pesticides residues, are expressed in parts per million (mg kg).

Photo original. Dr. Nicoleta Marin: S. V. F. S. Constanta Dolphins strended in12 - 13.04.2009

2. RESULTS AND DISCUSSION

Tabel no 1

Concentration of organochlorine pesticides analysed at dolphins strended between 2007 to 2009 from bubbler, expressed as mg/kg lipid weight. In parentheses is found detection limite of compounds. (ND = not detectable).

Dolphins strended Species/sex/ Strended date	αHCH (0,005)	Lindane (0,002)	βHCH (0,01)	DDT total (0,04)	Endrine (0,01)	Aldrine (0,01)	Hepta-chlor (0,01)	Dieldrine (0,01)	HCB (0,01)
1.*Tursiops.t.p.*Male 23.10.2007	0,024	0,082	2,227	20,09	ND	ND	ND	ND	ND
2. Phocena P.Unknoun sex.12.10.2007	0,106	0,074	6,533	10,944	0,180	ND	ND	ND	ND
3.*Tursiops t.p.* female 29.04.2008	0,002	0,005	0,418	38.8	0,260	ND	ND	ND	0,580
4 *Tursiops t.p.*Male 30.04.2008	0,043	0,121	5,50	27,65	ND	ND	ND	ND	ND
5*Tursiops .t.p.*Female 05.05.2008	0,02	0,01	1,25	8,99	ND	ND	ND	ND	ND
6.*Phocena P.*	0,025	ND	2,54	4,361	ND	ND	ND	ND	ND

Mascul 27.06.2008									
7.*Phocena P.*Female 04.07.2008	ND	ND	1,77	9,53	ND	ND	ND	ND	0,09
Dolphins strended Species/sex/ Strended date	αHCH (0,005)	Lindane (0,002)	βHCH (0,01)	DDT total (0,04)	Endrine (0,01)	Aldrine (0,01)	Hepta-chlor (0,01)	Dieldrine (0,01)	HCB (0,01)
8. *Delphinus D.* Female 20.07.2008	ND	ND	1,821	9,96	ND	ND	ND	ND	0,13
9. *Phocena P.* Female 23.07.2008	0,190	3,005	12,151	ND	ND	ND	ND	ND	ND
10. *Phocena P.* Male 02.09.2008	0,075	0,022	2,592	7,846	ND	ND	0,272	ND	ND
11.*Tursiops truncatus p.* Female.13.04.2009	0,034	1,380	10,75	ND	ND	ND	0,041	ND	0,074
12.*Phocena phocena* Male.13.04.2009	0,034	1,60	9,42	ND	ND	ND	ND	ND	0,072
13.*Phocena phocena* Male.13.04.2009	0,031	1,24	9,01	ND	ND	ND	ND	ND	0,072
14.*Phocena phocena* Female13.04.2009	ND	4,32	34,94	ND	ND	ND	ND	ND	0,382
15.*Phocena phocena* Female13.04.2009	ND	2,46	12,65	ND	ND	ND	0,727	ND	0,280
16.*Phocena Phocena male*27.05.2009	0,045	2,66	13,2	ND	ND	ND	ND	ND	0,14
17.*Phocena Phocena* female21.06.2009	0,050	0,64	7,98	ND	ND	ND	0,600	ND	0,32
18.*Tursiops truncates p.*male(Mark) 2.07.2009	ND	1,32	47,26	ND	ND	ND	0,045	0,07	0,13

- α HCH concentrations ranged from 0,002 to 0,190mg/kg lipid weight;
- gamma HCH concentrations ranged from 0,005 to 4,32 mg/Kg lipid;
- beta-HCH concentrations ranged from 0,418 to 12,151 mg/Kg lipid ;
- DDT total concentrations ranged from 4,361 to 47,26 mg/Kg lipid;
- Endrin concentrations ranged from 0,260 to 0,180 mg/Kg lipid;
- Aldrin- not detectable ;
- Heptaclor concentrations ranged from 0,041 to 0,727 mg/Kg lipid;
- Dieldrin not detectable ;
- HCB concentrations ranged from 0,072 to 0,580 mg/Kg lipid;

Tabel no.2.

Concentration polichlorutated biphenyl from strended dolphins between in 2007 to 2009 from bubller, expressed as mg/kg lipid weight. In parentheses is found detection limits of compounds.(ND= not detectable)

olphins strended Species/sex/ Strended date	PCB 28 (0,005)	PCB 52 (0,005)	PCB 101 (0,005)	PCB 153 (0,005)	PCB 138 (0,005)	PCB 180 (0,005)	PCB 31 (0,005)	PCB 118 (0,005)
Tursiops.t.p ale; 23.10.2007	0,180	0,632	0,565	1,153	ND	0,548	0,316	-
Phocena P. nknoun sex; .10.2007	ND	ND	ND	0,646	1,314	0,095	ND	-
ursiops t.p. emale; 29.04.2008	ND	0,35	0,24	1,34	1,13	0,43	0,23	-
Tursiops t.p ale; 30.04.2008	ND	0,390	ND	0,546	ND	0,906	ND	-
Tursiops t.p male; 05.05.2008	ND	ND	0,012	0,031	0,028	ND	ND	-
Phocena P. ale; 27.06.2008	ND	ND	ND	0,159	0,649	ND	ND	0,248
Phocena P male ; 04.07.2008	ND	ND	0,340	0,360	ND	0,17	ND	0,248
Delphinus Female; 07.2008	ND	ND	0,324	0,258	ND	ND	ND	0,248
Phocena P male; 23.07.2008	ND	0,252	ND	2,166	1,614	0,335	ND	-
.Phocena P. ale; 02.09.2008	ND	ND	ND	0,463	0,759	0,256	ND	-
.Tursiops t.p.; male; 3.04.2009	ND	0,074	0,039	0,242	0,333	0,752	0,016	0,184

2.*Phocena* *hocena* Male; 3.04.2009	ND	0,036	ND	0,133	0,249	0,045	ND	0,040
3.*Phocena* *hocena*Male.; 3.04.2009	ND	0,036	0,028	0,207	0,349	ND	0,039	0,112
4.*Phocena* *hocena* Female; 3.04.2009	ND	0,684	ND	1,74	ND	7,08	ND	ND
5.*Phocena* *hocena* Female; 3.04.2009	ND	0,824	0,279	1,81	3,43	ND	ND	ND
Dolphins strended Species/sex/ Strended date	PCB 28 (0,005)	PCB 52 (0,005)	PCB 101 (0,005)	PCB 153 (0,005)	PCB 138 (0,005)	PCB 180 (0,005)	PCB 31 (0,005)	PCB 118 (0,005)
6.*Phocena* *Phocena Male;* 7.05.2009	ND	0,06	0,268	0,33	ND	0,16	ND	ND
7.*Phocena* *Phocena* Female; 1.06.2009	ND	0,07	0,345	0,346	ND	0,200	ND	0,120
8.*Tursiops* *runcatus* .male;; Mark) .07.2009	ND	0,137	0,205	1,69	2,27	0,85	ND	ND

- PCB 28 concentration was faunded only in the first sample(0,180 mg/Kg lipid weight);
- PCB 58 concentrations ranged from 0,036 to 0,824 mg/Kg lipid weight ;
- PCB 101 concentrations ranged from 0,012 to 0,565 mg/Kg lipid weight;
- PCB 153 concentrations ranged from 0,031 to 2,166 mg/Kg lipid weight;
- PCB 138 concentrations ranged from 0,028 to 3,430 mg/Kg lipid weight;

- PCB 180 concentrations ranged from 0,045 to 7,080 mg/Kg lipid weight;

- PCB 31 concentrations ranged from 0,230 to 0,316 mg/Kg lipid weight;

- PCB 118 concentrations ranged from 0,040 to 0,248 mg /Kg lipid weight;

3.CONCLUSIONS

3.1. The highest concentration of organochlorine pesticides was registreted in lindane (4,320 mg/Kg lipid weight), beta HCH (12,151 mg.Kg lipid weight) and DDT s (47,260 mg/Kg lipid weight).

3.2 .PCBs concentrations ranged from 0,818 to 9,504 mg/Kg lipid weight.

3.3 This study shows that there is pollution in the marine environment with pesticides and PCBs and reduce the number of dolphins (strended) may be due them, the toxicity of these pollutants being able to cause reproductive problems, endocrine, affects the liver, kidney, pancreas, testes, and immune system, are neurotoxic and carcinogenic.

In this regard we draw an alarm to save the dolphin and the aquatic environment, through measures to be taken by all authorized institutions.

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PROBIOTICS: AN OPPORTUNITY FOR SWINE HEALTH AND PERFORMANCE

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Keywords: probiotics, metabolic activity, immunologic effects.

SUMMARY

Making a bibliographic study on probiotics has aimed to clear in what extent they can be an opportunity for health and performance in pigs.
The relationships of the microbiota with the host body is dominated by competitive production of energy and amino acids and from bacterial fermentation in the cecum and colon, with competitive inhibition of potentially pathogenic micro-organism and positive immunomodulation.
Intestinal dismicrobism occurs under the influence of biotic or abiotic stress factors and the balance is restored by adding the optimum combination of probiotics.
The qualities of the probiotics used in pigs are linked with the ability to survive along the digestive tract with high adhesion for colonization and benefic effects on the host organism and high stability after the first administration.
It is generally accepted that the stimulating effect of lactic bacteria is beneficial for their attachment to the intestinal lining that will protect animals from gastro-enteric infections.
In pigs, probiotics have the next effects: nutritional, sanitary, dis-metabolic effect for pathogens by disruption of enzymatic activity and secreting antibiotic substances (lactic bacteria) and general and local immunological effects. Evaluation of probiotics in animal nutrition is made by "in vitro" and "in vivo" tests in animals and by safety tests.
Effects of probiotics administered to pigs are correlated with increasing the weight gain, increasing reproductive performance in sows, boosting overall status and stress resistance in piglets and reducing prophilactico-curative utilization of antibiotics.

The relationships of the microbiota with the host-organism

The recent studies were finalized by the development of two conclusions:

- bacteria in the small intestine are competitive with the host for energy and amino acids, with losses in the use of glucose from amino acids over 6% and 15%, resulting ammonia, cadaverine and γ-cresol;
- in the cecum and colon, between 5 and 20% of energy derived from bacterial fermentation but at both levels, probiotics prevent development of potentially pathogenic microflora and contribute to local and general immunity, beneficial host organism (Budino,

2006; Gagnon, 2007; Handari, et al., 2008; Roşu and Falcă, 2007; Vamanu et al., 2002).

Quantitative and qualitative changes in bacterial microflora meet the following conditions:

- various metabolic nutritional disorders;
- trauma or aggression;
- chronic diseases through immunosuppression;
- emotional or physical stress;
- anti-medication use (antibiotics, sulphonamides or other chemotherapeutic agents);
- toxins in the environment, through water or food;
- some ingredients of "food additives", present in highly processed food.

Essential for successful treatment with probiotics is the need to live cultures of probiotics in animals. Recent studies defined probiotics as "natural bowel bacteria, which, after oral administration, are able to establish and possibly to colonize the digestive tract and maintain or increase the natural microflora of the digestive tract to prevent colonization by germ pathogens and to ensure the safety of food.

Microbiota source for piglets, their contact with the sow is a mother, and the speed of colonization of their gut microbes depends on environmental contamination and the administration of probiotics.

Significant changes are found in weaned piglets when the macrobiote formed by the *Lactobacillus* suddently reduces and a brutal development of coliforms, followed by the emergence of diarrhea.

In addition to stressors, very important for intestinal microflora composition and concentration, is the use of anti-infectiouse drugs that can select pathogens, and can inhibit probiotics, for “good flora” is more sensitive to anti-infectiouse drugs (Gaskins, 2008; Kritas, 2007).

The principal action that led to the use of probiotics in therapy, that the growth performance of pigs, based on the mechanism of competitive exclusion, probiotics complex process by which bacteria develop preclude unwanted pathogens. The mechanism underlying the beneficial use of crops to correct nutritional balance and health of pigs, especially in intensive farming (EC, 2003, 2007).This provides the host intestinal microbiota mainly blocking certain services and intestinal colonization by pathogenic bacteria, providing the first stimulus needed immunological functions locally and generally, contributing also to host nutrition by producing short-chain fatty acids, amino acids and vitamins.

The contribution which the intestinal microbiota without a host for efficient growth is also related to how best to use nutrients by swine,

turn-over regulation of mucus and epithelium, and detoxification of the bacterial cataboliții own, continuous production of cells with inflammatory role or immunity. Many management practices in raising pigs, could destabilize relations of cooperation between the microbiota and host intestinal inflammation may result in more or less severe and even significant disruption of normal growth.

These may be related to food management strategies liquid or semi-solid (powder or granules), including the use of anti-(antibiotics, sulphonamides or other chemotherapeutic agents), probiotics and digestive enzymes, including quality and quantity of water, given ad libitum "or the cycles related to the administration of food (Link şi Kovac, 2006; Scharek et al., 2005; Schiffrin and Blum, 2002).

Characteristics of probiotic bacteria can be summarized by the following qualities:

To be able to survive along the digestive tract following conditions:

a. the oral cavity lithic action of salivary lysozyme;
b. (sometimes under 3) and the time of resistance can be increased by including probiotics on food substrates protective;
c. the small intestine to resist bile juice action;

B. To allow satisfactory adhesion to the intestinal wall to avoid being eliminated by peristaltic movements and colonization to make for a sufficiently long to have a beneficial effect (Gaskins, 2008; Scharek et al., 2005).

The action of microorganisms in the digestive tract and their nutritional needs

Probiotics are defined as indigestible food ingredients that affect the host by stimulating beneficial selective growth and / or activity of a limited number of bacteria in the colon (Roşu and Falcă, 2007).

It is generally accepted that stimulation is beneficial lactic bacteria by attaching their intestinal mucosa, which will protect the animals from infection at this level.

Table 1

Metabolic activity of microbiota in the gut of pigs (Roșu și Falcă, 2007)

METABOLIC PROCESSES	BENEFICIAL ON HOST	AGENT INJURIOUS TO HOST
proteolysis	animoacds release of proteins in foods and in the endogenous	loss of amino
degradation of amino acids	reuse of ammonia in the synthesis of amino acids	excess ammonia
synthesis of amino acids	increasing use of cholesterol	toxic end product
transformation of bile acids and of sterols	preventing excess calcium absorption	degestibilitatii reduce fat
hydrogenation of fatty acids	uncertain benefits for coprophagy	zinc deficiency aggravated
		emphasizing the effect of any deficiencies
		increase nutritional requirements

It is generally accepted that stimulation is beneficial lactic bacteria by attaching their intestinal mucosa, which will protect the animals from infection at this level.

Effects of probiotic bacteria use

1. Direct nutritional effect, manifested by measurable performance improvement through breeding grounds (greater increases in weight, lower specific consumption, higher output).

These effects are achieved through the following mechanisms of action of probiotics:

a. decreases the intestinal pH, reduces the frequency of diarrhea in piglets;
b. stimulate production of endogenous enzymes;
c. predigests protein;
d. production of hydrogen peroxide, which activates the lactoperoxydasetiocyanat;
e. additional contribution of amino acids, vitamins and organic acids;
f. control the production of amines (antitoxins);
g. destruction of the anti-nutritive factors;
h. Change in metabolic costs for the digestive function of the animal.

2. Health Effect: probiotics help to strengthen the body's natural defense.

a. prevent intestinal colonization by pathogenic factors contributed to stress, (a drastic change in diet, antibiotherapy). This barrier effect which opposes the existing flora of a new embryo implantation can be explained by:

- competition on availability of nutrients as well as production by probiotic flora of metabolites with toxic effects on pathogens (volatile fatty acids);
- filling the sites of attachment of probiotic flora in digestive mucosal surface.

b. Reducing the translocations of pathogenic bacteria in the intestinal lymph nodes;

c. stimulate the body immunity by:

- raise antibodies (eg if *Lactobacillus plantarum* and *L. casei*, which increases the production of Ig A in the intestine);
- increased activity of macrophages.

3. Impaired metabolism of pathogens by increasing or inhibiting the activity of enzymes;
4. Secrete antibiotic substances by lactic acid bacteria cultures (Gaskins, 2008; Handari, et al., 2008; Link and Kovac, 2006; Roşu and Falcă, 2007; Vamanu et al., 2002).

Reducing the prevalence of pathogenic microorganisms in the digestive tract contributes to improving the nutritional effect of probiotics.

Evaluation of probiotics administered pigs

Taxonomic classification of probiotic strains is in accordance with internationally recognized nomenclature and contained in:

1. Aproved List of Bacterial Names (Int. J. Syst. Bacteriol., 1980, 30, 225-420);
2. Validation List. (Int. J. Syst. Bacteriol. Prior to 2000) (EC 2003, 2007).

The main tests "in vitro" to the state probiotic strains are:

a. resistance to gastric pH decreased;
b. resistance to bile salts;
c. adhesion to the surface mucosa (cell culture);
d. antimicrobial activity against pathogens;
e. bility to reduce pathogen adhesion to mucosal surface;
f. resistance to the action of intestinal digestive juices.

Tests in vivo can confirm the results obtained "in vitro" and mechanisms of action of probiotic strains.

Main results of efficacy studies relate to:

- reduce morbidity and mortality;
- rapid recovery after illness;
- feed conversion;
- average daily increase;
- food consumption.

Tests are carried out generally as randomized, double blind, linked to quantify adverse effects on the statistical analysis (Vamanu el al., 2002; EC, 2003, 2007).

. Aspects of the use of probiotics in animal husbandry.Digestive tract microbiotics in pigs

The major objectives of the use of probiotic preparations in the growth of pigs are:

- improves animal growth rates;
- increasing the food conversion rate;
- improves the quality of animal, carcass grades default;
- increasing the digestive efficiency in eliminating pathogenic flora;
- major contribution in achieving organic meat (organic).

The pigs, probiotics can be recommended by bacterial colonization method (in newborns), for prophylaxis of diarrhea digestive syndromes, before weaning and after weaning (the youth), to prevent the effects of sorting related stress, vaccinations, ration changes (any age) to treat infectious disbacterioses and proliferation of toxic effects arising from the use of oral therapy with anti-infectiouse drugs as immunostimulators and growth promoters (Vamanu el al., 2002).

The EU has approved 13 probiotics as food additives for pigs, which they present in order to ration the amount of microorganisms accepted pigs, the predominant genera: *Enterococcus, Lactobaccillus, cerevisae Bifidobacterium, Bacillus, Streptococcus* (EC, 2003, 2007). Related to age pigs, administration of probiotics in piglets after birth and is recommended around the weaning period, when hypothrepsia risk after stressful activities (castration, tail docking and corner etc.), for prophylaxis and diarrheal symptoms. In older pigs is recommended to combat sorting stress, transport, vaccination, and after treatment with antibiotics or if qualitative and quantitative changes in the structure of the ration (EC, 2007).

The effects of probiotic bacteria use on swine

Decreases the duration and intensity of diarrhea in piglets syndromes profilactico-healing using probiotics is based on several mechanisms.

The first mechanism of action is blocking the receptor cell in the entherocyte, preventing adhesion and invasion of bacteria and viruses.

Coverage of the second mechanism of action would be possible amplification of immune response by probiotics, increased secretion of Ig A, but no concrete data on the rate of stimulation.

There is a third hypothesis involving probiotics in stimulating synthesis of glycosilated mucines, which confers protection of the entherocytes by the "anti-adhesion" effect, against viruses and pathogenic bacteria.

There is a fourth case which refers to the ability of probiotics to inactivate viral particles and prevent cell invasion by inducing lactic acid.

Possible explanations for local and general effects of the use of probiotic bacteria in certain digestive infections, should be related to a positive immunomodulation of the host organism, while competitive exclusion at the sites of adhesion.

The largest positive response to probiotics appears when administered to suckling and recently weaned piglets.

With over 40 models of feeding are used bacterial probiotics was observed in 2/3 of cases increased particularly the increase in weight (Budino, 2006; Estienne, 2005; Handari et al., 2008; Roşu and Falcă, 2007).

testing two groups of probiotics that contain genous *Enterococcus*, *Bacillus* and *Sacharomyces* several batches piglets in the week after weaning, have found that lots of animals that received probiotic there was no case of diarrhea that may require treatment.

These results attest that there is a positive effect of probiotics used in the health status of piglets immediately after weaning (Scharek et al., 2005; Schiffrin and Blum, 2002).

In recent years the positive effects (not always statistically significant) of probiotics have been demonstrated to improve feed intake, increase weight, early weaning, decreased diarrhea, reduce the number of coliforms in the faeces and reduce the need for antibiotic treatment (Gaskins, 2008; Handari et al., 2008; Schiffrin and Blum, 2002).

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INTENSIVENESS AND EXTENSIVENESS OF INTESTINAL PARASITE ELEMENTS IN FOXES FOR FUR, BRED UNDER INTENSIVE SYSTEM AND THE RISK OF DISEASE TRANSMISSION IN HUMANS

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Key words: intestinal infestation, zoonotic risk, fox for fur

SUMMARY

Investigations were carried out during 2005-2007 on foxes for fur, bred under intensive system, in order to study the intensiveness (EPG) and extensiveness (E%) of intestinal parasites and the risk of their transmission in humans. Therefore, we have taken coprological samples from foxes according to age and analysed them by qualitative (Willis) and quantitative (Mc. Master) flotation methods. We have also studied epidemiological case studies and data were introduced in tables and expressed graphically. The obtained results have shown the presence of protozoa from *Cryptosporidium* genus (EPG: 50-300) in 53,33 % of the studied samples, *Isospora* genus (EPG: 50-100) in 13.33%, nematodes from *Ancylostoma* genus (EPG: 50-150) in 20%, *Uncinaria* genus (EPG: 50-100) in 6.66% and *Toxocara* genus (EPG: 50-150) in 10.00%. The microscopic pictures were photographed with a digital camera.
The value of EPG shows a reduced infestation, typical of the subclinical evolution or the quality of carriers and removers of invasion elements, which is sufficient for providing human contamination and disease.

Foxes for fur are bred under a closed intensive system due to the fact that special conditions for feeding, microclimate and exploitation adapted to the sensitivity of these carnivores, are needed. Any discrepancy that appears in the breeding technology reflects on the quality of the fur thus having a negative impact upon the final economical purpose. Adding into the food the slaughterhouse residues that have not received a thermal treatment expose the foxes to subclinical parasitary infestations with a risk of being transmitted to the caretakers and to the processors. Generally speaking, the intestinal parasitic phauna of the foxes resembles the one that can be encountered to other wild and domestic carnivores (Gundlach et al, 2004, Cisek et al, 2004). These investigations have been completed aiming at studying and at determining the extent of the invasive intestinal elements on fur foxes and the risk of their transmission to the human being.

1. MATERIAL AND METHODS,

The research took place between 2005-2007 on a state property, on a number of fur foxes belonging to the polar fox type, and the blue fox type that have been bred intensively. In order to achieve this study there have been three series of samples taken from feces and these samples have been examined through the quality flotation method (Willis) and through the quantity flotation method (McMaster). The analysis of the extensivity (E%) and of the intensivity (OPG) of the intestinal parasites genus in fox has been made once a year, before the animals were sacrificed for their fur and them were analyzed for the whole time interval that was subjected to study. The data were included in tables and the results have been expressed graphically. The identification of the parasitic elements has been made using the Motic, oc. 10 x ob. 10, 20, 40 microscope; the microphotography was realized using a digital photo camera.

2. RESULTS AND DISCUSSION,

The coproscopic results revealed the presence of the parasitic elements belonging to the *Cryptosporidiun* protozoan type (fig. 1.) and *Isospora* (fig. 2.) and to the *Ancylostoma* nematodes type (fig. 3.), and *Toxocara* (fig. 4.). *Uncinaria*.

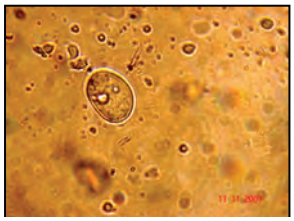


Fig. 1. *Cryptosporidium spp.* oc. 10 x ob. 40

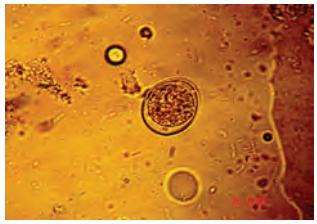


Fig. 2. *Isospora spp.* immature ovocyst oc. 10 x ob. 40

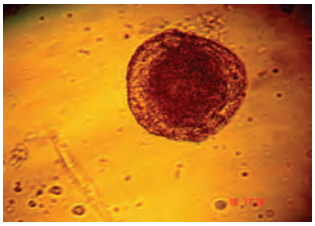


Fig. 3. *Ancylostoma caninum*, egg oc. 10 x ob. 20.

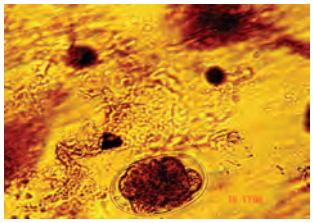


Fig. 4.*Toxocara canis:* egg. Oc. 10 x ob. 20

The results of the coproscopic examinations regarding the intensiveness and the extensiveness of the invasive intestinal elements in adult foxes for fur in 2005 can be found in Table 1.

Table 1

Intensiveness and (OPG) extensiveness (E%)
of the intestinal parasitic elements in adult foxes (oct. 2005)

Nr crt	Protozoans				Cestodes		Nematodes					
	Isospora		*Cryptosp.*				*Ancylostoma*		*Uncinaria*		*Toxocara*	
	OPG	E%	OPG	E %	OPG	E %	OPG	E%	OPG	E%	OPG	E%
1.	0	10	0	10	0	0	0	10	0	10	0	20
2.	0		100		0		50		0		0	
3.	50		0		0		0		100		0	
4.	0		0		0		0		0		150	
5.	0		0		0		0		0		0	
6.	0		0		0		0		0		0	
7.	0		0		0		0		0		50	
8.	0		0		0		0		0		0	
9.	0		0		0		0		0		0	
10.	0		0		0		0		0		0	

From the presented data, it can be observed the fact that the protozoans are dominant within the intestinal biocenosis with a 20% extensiveness and a 50-300 OPG, which suggests a weak intensiveness of the infestation. Also, the *Toxocara* type is present as well in 10% of the samples with a OPG-100 and *Uncinaria* with a OPG-50. These aspects reveals the fact that youth, in the first months of life, went through an enteral disease caused especially by *Cryptosporidium* and *Isospora* and they have been transplacentary infested with *Toxocara* and *Uncinaria* in the intrauterine time interval. The dynamics of the invasive elements in adult foxes for fur in October 2005, before the animals were sacrificed for fur is given in fig. 1.

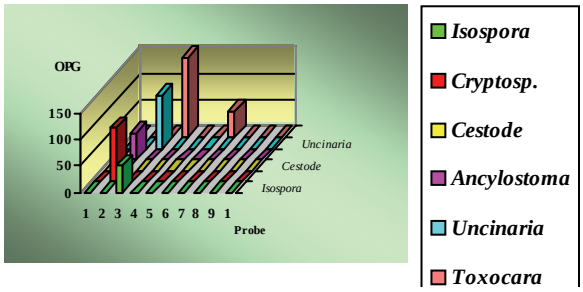


Fig. 1. The intensiveness dynamics (OPG) of the intestinal parasitic elements in foxes for fur in October 2005

From the presented data, it can be observed the fact that the general extensiveness of the intestinal parasitic elements in adults was of 60%. The largest intensiveness was observed in the Toxocara type with a OPG value: 150, followed by *Uncinaria* and *Cryptosporidium* with a OPG: 100. There were no invasive elements belonging to the cestodes type. These values suggest the presence of the portage phenomenon that contributes to the perpetuation of the species and their transmission to the youth. The coproparasitic results obtained from the fox youths are shown in Table 2.

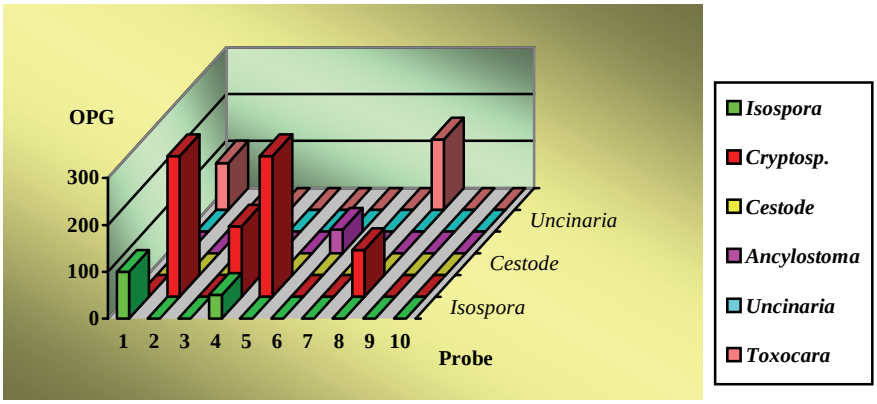
Table 2

Intensiveness (OPG) and extensiveness (E%)
of the intestinal parasitic elements in young foxes (oct. 2005)

Nrcrt	Protozoans				Cestodes		Nematodes					
	Isospora		*Cryptosp.*				*Ancylostoma*		*Uncinaria*		*Toxocara*	
	OPG	E%	OPG	E%	OPG	E%	OPG	E%	OPG	E%	OPG	E%
1.	100	20	0	40	0	0	0	10	0	0	100	20
2.	0		300		0		0		0		0	
3.	0		0		0		0		0		0	
4.	50		150		0		0		0		0	
5.	0		300		0		0		0		0	
6	0		0		0		50		0		0	
7	0		0		0		0		0		0	
8	0		100		0		0		0		0	
9	0		0		0		0		0		150	
10	0		0		0		0		0		0	

From the Table 2 data it can be observed the fact that the *Cryptosporidium* type is present in 40% of the samples with a value of the OPG: 100-300. There were no cestodes eggs found. The nematodes were identified in 10% the *Ancylostoma* type, and in 20% of the samples, the *Toxocara* type, with a variable OPG of 50-150, expressing a minimum level of infestation having a subclinic and portage character. The intensiveness dynamics of the invasive elements in young foxes for fur in 2005, is given in fig. 2.

Fig. 2. The intensiveness dynamics (OPG)of the intestinal parasitic elements in young foxes for fur in October 2005.



It can be observed the fact that the general extensiveness of the invasive elements from the examined samples is of 90% (9 samples out of 10 presented parasitic elements). Protozoans were present in 60% of the analyzed samples, and the nematodes in 30% of the samples. The results of the coproscopic examinations regarding the intensiveness and the extensiveness of the invasive intestinal elements in young foxes for fur in November 2006 (at the beginning of the season when animals are sacrificed for fur) can be found in Table 3.

Table 3

Intensiveness (OPG) and extensiveness (E%)
of the intestinal parasitic elements in young foxes (nov. 2006)

Nr crt	Protozoans				Cestodes		Nematodes					
	Isospora		*Cryptosp.*				*Ancylostoma*		*Uncinaria*		*Toxocara*	
	OPG	E%	OPG	E%	OPG	E%	OPG	E%	OPG	E%	OPG	E%
1.	0		100		0		0		0		0	
2.	0		100		0		50		0		0	
3.	0		50		0		0		0		0	
4.	0		150		0		0		50		0	
5	0	0	250	70	0	0	0	30	0	10	50	10
6.	0		100		0		50		0		0	
7.	0		0		0		0		0		0	
8.	0		0		0		50		0		0	
9.	0		0		0		0		0		0	
10.	0		300		0		0		0		0	

The data presented in Table 3 show the fact that the *Cryptosporidium* type was identified in 70% of the analyzed samples with a reduced variable intensiveness (OPG: 50-300). Nematodes were present in 30% of the samples, the *Ancylostoma* type and in 10% of the

samples, the *Toxocara* and *Uncinaria* types. The coproscopic results obtained in 2006 reveal an extensiveness of the protozoans of 70%, more important than in 2005.

The intensiveness dynamics of the intestinal parasitic elements in foxes for fur in November 2006, is given in fig. 3.

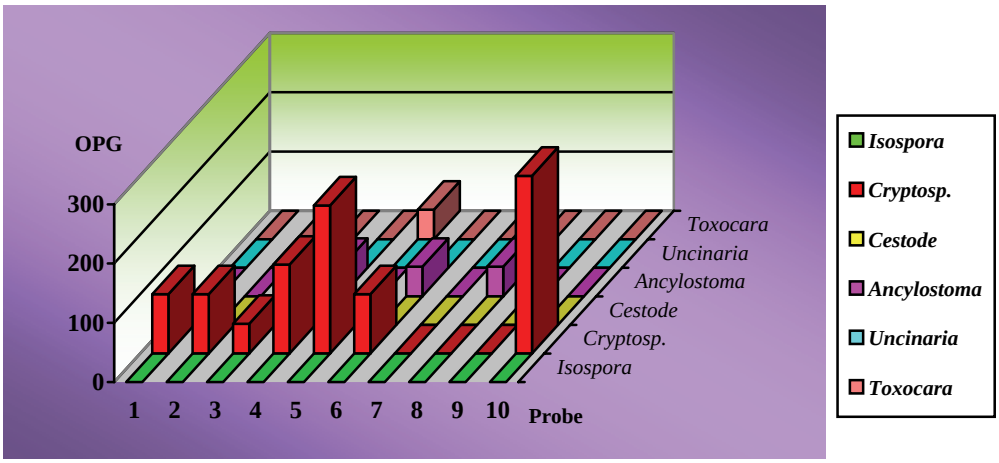


Fig. 3. The intensiveness dynamics (OPG)of the intestinal parasitic elements in young foxes for fur in November 2006

The results of the coproscopic examinations regarding the intensiveness and the extensiveness of the invasive intestinal elements in young foxes for fur in November 2007 can be found in Table 4.

Table 4

Intensiveness (OPG) and extensiveness (E%) of the intestinal parasitic elements in young foxes (nov. 2007)

Nr. crt	Protozoans				Cestodes		Nematodes					
	Isospora		*Cryptosp.*				*Ancylostoma*		*Uncinaria*		*Toxocara*	
	OPG	E%	OPG	E%	OPG	E%	OPG	E%	OPG	E%	OPG	E%
1.	50		100		0		0		0		0	
2.	0		0		0		0		0		0	
3.	0		50		0		0		0		0	
4.	0		150		0		150		0		50	
5	0	10	0	60	0	0	0	30	0	0	0	10
6.	0		100		0		50		0		0	
7.	0		0		0		0		0		0	
8.	0		100		0		50		0		0	
9.	0		0		0		0		0		0	
10.	0		50		0		0		0		0	

From the data presented in Table 4 it can be observed the fact that the extensiveness of the parasitic elements is of 80% (8 out of 10

samples were positive), the extensiveness of the *Cryptosporidium* type remained elevated, of 60%, with a OPG 50-150; the *Ancylostoma* type has an extensiveness of 30% which represents an increase in comparison to the previous years, and a minimum intensiveness, OPG: 50-150. The *Toxocara* type was present in 10% of the samples with OPG: 50. The dynamics of the intestinal parasitic elements in young foxes for fur in November 2007, is given in fig. 4

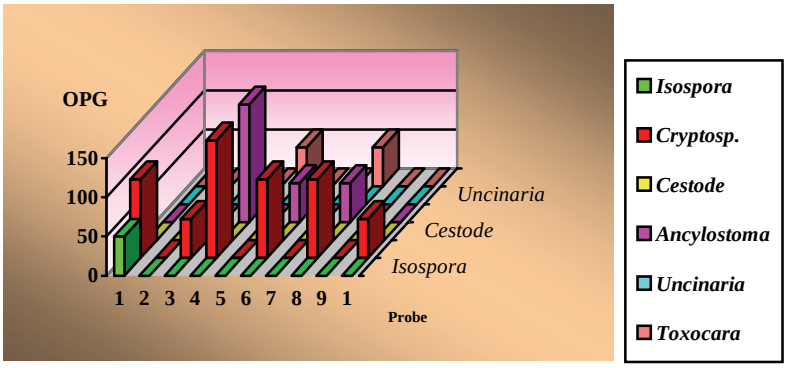


Fig. 4. The intensiveness dynamics of the intestinal parasitic elements in foxes for fur in November 2006.

The analysis of the dynamics of the intestinal invasive elements in youth reveals the dominance of the *Cryptosporidium* type in the three consecutive years, being found in 60% of the analyzed samples in 2007. The extensiveness analysis of the young foxes for fur in 2005-2007 is shown in Table 5.

Table 5

The Extensiveness (E%) of the intestinal parasites types in young foxes for fur in 2005-2007

Nr. crt	Parasite type	Analyzed samples	Positive samples	E(%)
1.	*Isospora*	30	4	13,33
2.	*Cryptosporidium*	30	16	53,33
3.	*Cestodes*	30	0	0
4.	*Ancilostoma*	30	6	20,00
5.	*Uncinaria*	30	2	6,66
6.	*Toxocara*	30	3	10,00

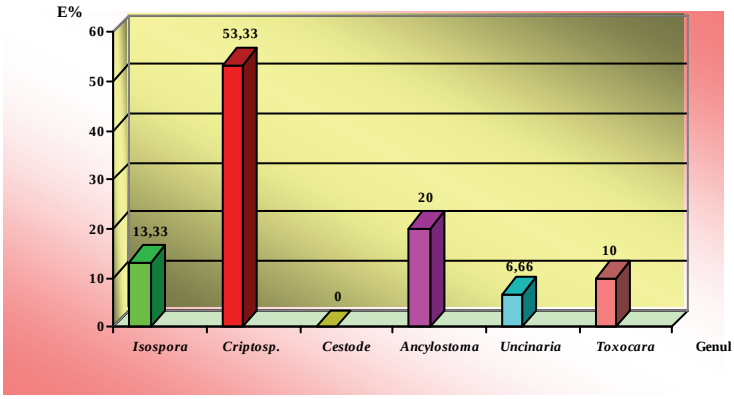


Fig. 5.The Extensiveness (E%) of the intestinal parasites types in young foxes for fur in 2005-2007

The elevated incidence of the *Cryptosporidium* oocysts in youth is concerning due to their transmission among the animal community and the cross-transmission from animal to humans and from humans to animals. Cryptosporidiosis is a severe parasitic zoonosis and, in certain conditions it has the character of a occupational disease for the holders, breeders and caretakers of the ranch or apartment animals, and for the medical veterinary stuff (Szell et al, 2004). The association of cryptosporidiosis with bacterial and viral infections contributes to the complication of the clinical evolution sometimes having irreversible effects for the infested subject. *Cryptosporidium spp.* is also an opportunist that develops very well in weaken organisms, debilitated, imunodepressed, amplifying the pathogen aggression mechanisms, more severe and more complex than in the case of the unique etiology.

The minimum intensiveness reflects the subclinical and portage state of the invasive elements that do not determine clinical expressions, thus being more severe due to their unobserved passage. The association and the coexistence of more types of parasites: *Cryptosporidium*, *Isospora*, *Ancylostoma*, *Toxocara* and *Uncinaria*, in the same multidimensional niche – the small intestine and the utilization of the same nutritional support conducts to the selective spoliation of the nutritive elements by the parasites in the detriment of the host organism and the parasitic pollution of the environment with parasitic elements (Saed et al, 2006). In these circumstances the human contamination and

infestation constitute a real risk (Antolova et al, 2004, Iacob, Olimpia, 2006).

3. CONCLUSIONS,

This study revealed the presence within the dynamics of the intestinal parasitic elements in foxes for fur that have been bred in an intensive system with a closed circuit.

3.1. The paraclinic examinations lead to the identification of the parasitic elements belonging to some protozoans and nematodes with variable extensiveness (E%): *Cryptosporidium*: 53,33%, *Isospora*:13,33%, *Ancylostoma*: 20%, *Toxocara*: 20% and *Uncinaria*: 6.66%

3.2. The intensiveness (OPG) of the infestation was minimum, the OPG ranging in value between 50-300, which defines the subclinic infestation, the youth being more intensively parasited with protozoans, and the adult foxes with nematodes.

3.3. The invasive parasitic elements identified in the intestines of the foxes for fur constitute a major risk for the caretakers of the animals and for the operators due to their transmission to humans determining severe zoonosis.

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ASSESSMENT OF THE QUALITY OF INTRAVENOUS PYELOGRAPHY AND OF THE IOHEXOL DYNAMICS IN CAT

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Key words: pyelography, iohexol, cat, radiography

Summary

There was assessed the quality of pyelograms in the time frame of 0 to 120 minutes after intravenous injection of 600mg/kg iohexol solution. The time of every radiography is strictly recorded and visibility of organic structure (renal cortex, calices, renal pelvis, ureters, urinary bladder) is evaluated. The study revealed that first adequate pyelography is obtained after 4 minutes and 49 seconds and the last one is obtained after 27 minutes and 46 seconds. The last radiography to suggest the presence of contrast medium is obtained after 1 hour, 55 minutes and 13 seconds. Regarding the position of the animal during x-ray exposure, it is recommended to execute lateral radiography and also dorso-ventral radiography in order to better assess full length of ureters. During examinations there were difficulties in evaluating the full length of one ureter that was behind the colic fecal mass, in lateral view, supporting the idea of two positions pyelogram.

Radiography of the abdomen after intravenous injection of contrast medium results in pyelography, ureterography, cystography. The use of such contrast medium is a good method of assessment of urinary system. Indications of radiography with contrast medium include assessment of kidney considering the regular appearance, smooth outlines, size, position, equal filtration and flow; ureters are assessed for size, smoothness regularity and symmetrical appearance; the urinary bladder is assessed for regular smooth appearance and complete voiding. The objective of this study was to find the best time post injection in a superficial vein, for obtaining good images that can be used for assessment of the urinary system of the cat. A second objective is to find out after how much time the iohexol solution becomes visible and for how long it can be identified on radiographs.

1. MATERIAL AND METHOD

The subject of the study is an adult cat, in good shape, clinically sound, 3.85 kg of weight, female and spayed. The contrast medium is iohexol, 350 mg/ml, using 7 ml of solution (Ominpaque) resulting in 636.36 mg/kg of body weight (Mahawar J.K., 2005). The vein used is

the cephalic vein. The cat is under general anesthesia. The radiographic machine (Phillips Practix 33 plus) is set for 50 kv and 5 mAs. A chronograph is used to establish the exact time of each radiography made. Radiographs are made in dorso-ventral position and in lateral position. After rapid intravenous injection the first radiography is made after 27 seconds. The 2nd is made after 1 minute(m) and 15 seconds(s), the 3rd after 4m49s, the 4th after 9m17s, the 5th after 17m20s, the 6th after 27m46s, the 7th after 47m55s, the 8th after 1 hour 1m18s, the 9th after 1 hour 10m52s, the 10th after 1 hour 26m44s and the 11th after 1 hour 55m13s. The number of radiographs is not arbitrary; it follows the presence of iohexol solution on images. All radiographs with contrast medium include assessment of kidney considering the regular appearance, smooth outlines, size, position, equal filtration and flow; ureters are assessed for size, smoothness regularity and symmetrical appearance; the urinary bladder is assessed for regular smooth appearance and filling status.

2. RESULTS AND DISCUSSION

The 1st radiography made after 27 seconds has no suggestion of a contrast medium presence. Both kidneys are visible. The 2nd radiography reveals the first sign of contrast medium in renal cortex and calices. The 3rd radiography shows the renal cortex, calices, renal pelvis, ureters in full length clearly visible(Fig.1)(Ruth Dennis, 2001), and urinary bladder is beginning to fill with contrast medium. Note that the uretero-cystic junction is clearly visible (Fig. 2). The 4th, the 5th and the 6th radiograms have similar aspect with the urinary bladder with better contrast as the quantity of iohexol elevates. From this point, all radiograms show poorer contrast. In the 7th radiography there is very good image of the bladder but ureters become less visible along with renal cortex and calices. The initial part of the ureters has similar contrast with the previous radiography. The 8th radiography shows comparative image. The 9th radiography reveals that the ureters are no longer visible in full length; the initial part of the ureter still has better contrast on the image but is less visible compared with the previous radiography. The bladder is now very visible, full with contrast medium(Anthony Carr, 2005). The 10th radiography reveals that the bladder and the ureters are just partially visible with very poor contrast. The last radiography (11th) shows the poorest contrast of all, with ureters very hard visible only in the initial part. The bladder is the only very visible organ (see table 1).

In every lateral radiography the ureters have different initial track. The uretero-cystic junction is visible only in lateral view (Fig.2), this being the choice radiography for assessing this zone. In lateral position there is possible that the fecal mass to obstruct good visibility of one ureter (Fig.2). This is less common in dorso-ventral position.

Table 1

Table showing the image quality for kidney, ureters and urinary bladder for each pyelography. The image quality is of 3 types: no contrast, good and poor. There is also recorded the time of every radiography in hour (h), minutes (m), seconds (s).

NUMBER	ORGAN	TIME	IMAGE QUALITY
1	kidney	**27s**	No contrast
	ureters		No contrast
	urinary bladder		No contrast
2	kidney	**1m15s**	Poor contrast
	ureters		No contrast
	urinary bladder		No contrast
3	kidney	**4m49s**	Good contrast
	ureters		Good contrast
	urinary bladder		Poor contrast
4	kidney	**9m17s**	Good contrast
	ureters		Good contrast
	urinary bladder		Good contrast
5	kidney	**17m20s**	Good contrast
	ureters		Good contrast
	urinary bladder		Good contrast
6	kidney	**27m46s**	Good contrast
	ureters		Good contrast
	urinary bladder		Good contrast
7	kidney	**47m55s**	Poor contrast
	ureters		Poor contrast
	urinary bladder		Good contrast
8	kidney	**1h01m18s**	Poor contrast
	ureters		Poor contrast
	urinary bladder		Good contrast
9	kidney	**1h10m52s**	Poor contrast
	ureters		Poor contrast
	urinary bladder		Good contrast
10	kidney	**1h26m44s**	No contrast
	ureters		Poor contrast
	urinary bladder		Good contrast
11	kidney	**1h55m13s**	Poor/no contrast
	ureters		Poor/no contrast
	urinary bladder		Good contrast

If the time of the first and the last good contrast image can be approximated it means that the best time for this kind of radiography is

between 5 minutes and 30 minutes after intravenous injection (Gary D. Norsworthy, 2006).

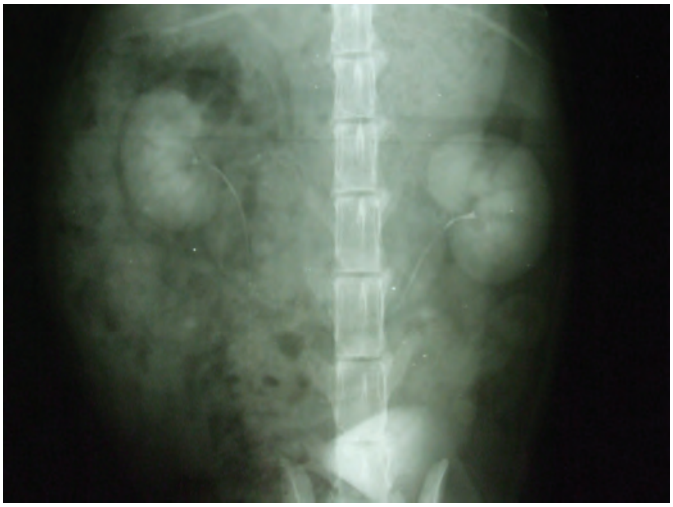

Fig.1. very good contrast on a dorso-ventral radiography. The entire kidney is visible along with ureters in full length and the filling bladder. Note the very visible initial part of ureters but not the uretero-cystic junction. The renal cortex and medulla are also very clear visible.

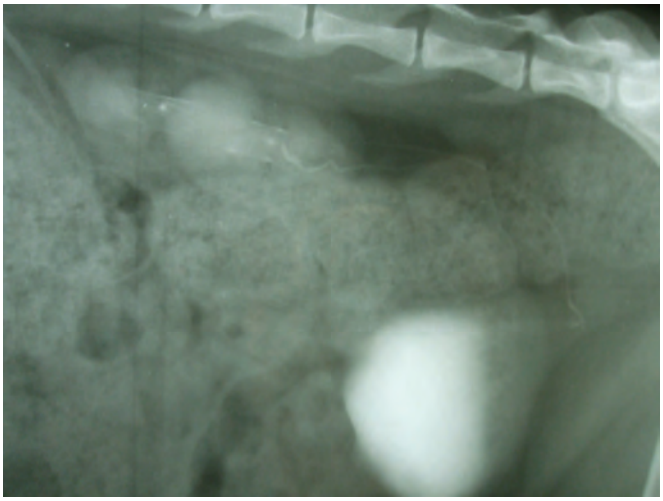

Fig.2. radiography with very good contrast, in lateral view. Kidneys and ureters are very visible, the uretero-cystic junction is assessable. The fecal mass is obstructing the assessment of the further ureter.

3. CONCLUSIONS

3.1. The best time frame for good quality pyelography in order to make an optimal assessment is between 5 minutes and 30 minutes after intravenous injection of iohexol solution.

3.2. There were less relevant and of poorer quality the radiographs made between 30 minutes and one hour post injection.

3.3. Even after two hours post injection, the iohexol solution is visible at the initial part of the ureters.

3.4 It could be of great help that before any examination the animal to receive an enema that will clear out the feces from the terminal part of the colon. By this procedure we could eliminate one cause of poor quality images considering that the fecal mass can interfere with the ureteral image.

3.5. In order to assess the full length of the ureters radiographs should be made in dorso-ventral position but also in lateral position. In dorso-ventral position there is a very good image of both ureters for most of their length but the last part is obstructed by the urinary bladder. In lateral position this part is clearly visible and assessable.

3.6. Between 4 and 5 minutes after injection the renal cortex, calices and renal pelvis are visible and assessable. There are also visible the ureters in full length and also the urinary bladder which begins to fill with contrast medium.

3.7. The iohexol dose of 600mg/ml, 350mg/ml provides good contrast pyelography,

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ADVANTAGES AND LIMITS OF TONOMETRY AT THE DOG

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Key words: intraocular pressure, aqueous humour, tonometer, tonometry

SUMMARY

The intraocular pressure (intraocular tension, ophthalmotonus) at the dog is dependent on the equilibrium between the production and drainage of the aqueous humour. Its normal values are between 16 and 22 mmHg, with variations correlated to the individual (race, age, animal`s behavior, day period) or the type of tonometer used for its measurement. (3, 6, 7, 9, 10, 16)
The grown intraocular pressure is present at the great majority of primary and secondary glaucoma cases. The low intraocular pressure is frequent at uveits and during anesthesia, generally.
Presently, we use the following tonometers for the measurement of IOP in the practical ophthalmologic veterinary medicine: Schiotz, Perkins Mk2, Tono-Pen XL and Tono vet.
The measurement technique of the intraocular pressure at the dog depends on the type of tonometer. So as to accomplish the tonometry, the way of contention of the animal can be mechanic or by using drugs. Depending on the type of tonometer, the measurement of the IOP can be unique or multiple (successive determinations with the display of their average value). (7, 10, 13, 14)
The authors present in this paper a comparative study of the advantages and limits of the different types of tonometers used for the measurement of the intraocular pressure at the healthy dogs. They consider the main features of each tonometer type and make an evaluation on a scale from 1 to 10.

The aqueous humour produced at the level of the ciliary processes is a plasma ultrafiltrate with a refraction index of 1,335 that ensures the cornea and crystalline nutrition, taking over the products obtained as a result of the metabolism at the level of the eyeball. It contributes to the maintenance of the ocular ball`s shape and by its transparency, it ensures the bright radiation passing towards retina. (5, 7, 8, 9, 15)

The ciliary processes perform the control of intraocular pressure through the processes of active secretion, ultrafiltration and passive diffusion. The ciliary muscle and the iris influence the drainage of the aqueous humour at the level of iridocorneal angle. (7, 8, 9, 10, 12, 15, 16)

The intraocular pressure at the dog (table 1) shows variations depending on the day period (IOP value is bigger in the morning) and

age (it is lower at adults, as compared to the young ones). Hypovolemic and cardiogene shocks together with dehydration have as result the decrease of the IOP value. Some behavior typologies (dogs pulling the leash) influence the IOP value and this one is important in ocular affections that evolve together with its growing (glaucoma). (8, 10, 15, 16)

Tabel 1

The normal values of the ocular tension (Gelatt 2007)

Tonometry through applanation			
		Tono-Pen	MacKay-Marg
Dog	Average value(mm	16,8 ± 4,0	17,1 ± 3,9
	limits (mm Hg)	9 – 24	9 – 25
	limits (mm Hg)	9 – 31	12 – 32

Most anaesthesics and tranquilizers lower the intraocular pressure but ketamine determines its evolution. The ocular ball's inflammations (surgical or infectious) determine the decrease of aqueous humour production and intraocular pressure, implicitly. This paper presents the advantages and the limits of tonometry applied to the dog because the growth of the IOP value in case of glaucoma and hydrophtalmy affects the components of the ocular ball. (2, 7, 9, 10, 14, 15, 16)

1. MATERIAL AND METHOD

In our clinic, Centrovet Bucharest, we studied a number of 62 cases (dogs between 2 and 10 years old, of different sexes and breeds), from January until August 2009. These dogs didn't have clinical ocular pathology.

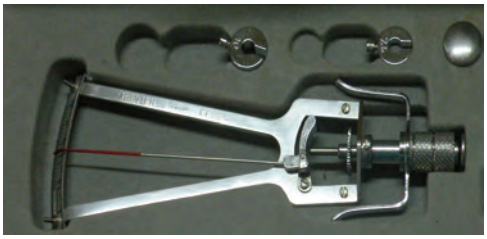
The intraocular pressure of these patients had been measured with different types of tonometers (Schiotz, Perkins MK2, Tono-Pen XL and TonoVet), twice a day, in the morning and evening, during 5-7 or 10 days. The animals have been subjected to rest for 30 minutes before IOP measuring.

The purpose of the study was to point out advantages and limits of the IOP measurement techniques at dogs, using different types of tonometers.

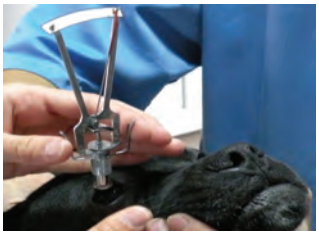
The measurement of ocular pressure was made at the free or restrained dog. The contention was manual, mechanical (muzzle) or chemical (local anaesthesia, sedation, general anaesthesia).

Schiotz tonometer (picture 1) is a mechanic tonometer of contact that uses weights of 5,5g, 7,5g, 10g and 15g for the measurement of IOP.

After the measurement of intraocular pressure (picture2), the achieving of IOP value needs the use of a conversion table (table 2) in order to correlate the used value with the value read on the tonometer scale and ocular pressure.

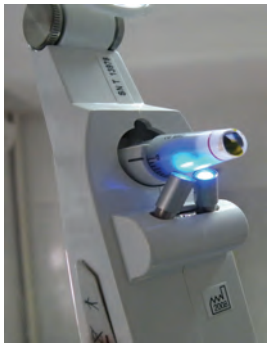


Picture 1 Schiotz tonometer

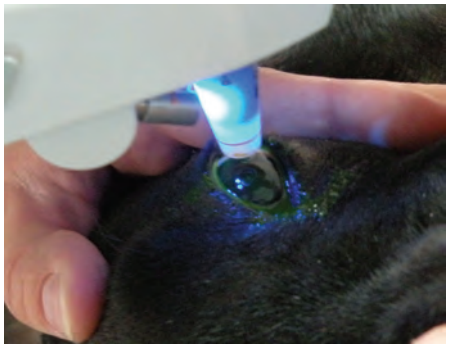


Picture 2 IOP measurement using Schiotz tonometer

Perkins MK2 tonometer (picture 3) is an optic-mechanical-electrical tonometer of contact, of Fick-Maklakov applanation that uses a system of calibrated weights, a system of prisms and its own source of light in order to obtain a direct determination of the intraocular pressure. It needs preliminary instillations with flourescein. Pressure determination (picture 4) is made by positioning an image of some semicircles which are produced by the prisms system and visualized through an integrated magnifying glass. It needs a periodical checking.



Picture 3 Perkins Tonometer Mk2



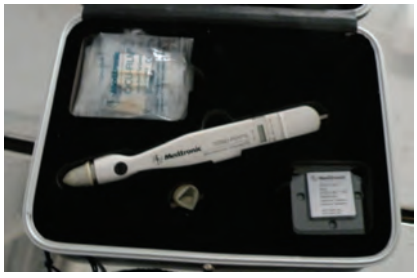
Picture 4 IOP measurement using Perkins Mk2 tonometer

Tabel 2

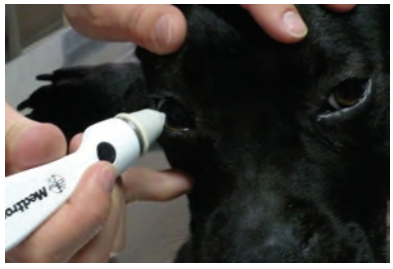
Table of conversion for Schiotz tonometer

Scale value	Used weight			
	5,5 g	7,5 g	10,0 g	15,0 g
0,0	41,5	59,1	81,7	127,5
0,5	37,8	54,2	75,1	117,9
1,0	34,5	49,8	69,3	109,3
1,5	31,6	45,8	64,0	101,4
2,0	29,0	42,1	59,1	94,3
2,5	26,6	38,8	54,7	88,0
3,0	24,4	35,8	50,6	81,8
3,5	22,4	33,0	46,9	76,2
4,0	20,6	30,4	43,4	71,0
4,5	18,9	28,0	40,2	66,2
5,0	17,3	25,8	37,2	61,8
5,5	15,9	23,8	34,4	57,6
6,0	14,6	21,9	31,8	53,6
6,5	13,4	20,1	29,4	49,9
7,0	12,2	18,5	27,2	46,5
7,5	11,2	17,0	25,1	43,2
8,0	10,2	15,6	23,1	40,2
8,5	9,4	14,3	21,3	38,1
9,0	8,5	13,1	19,6	34,6
9,5	7,8	12,0	18,0	32,0
10,0	7,1	10,9	16,5	29,6
10,5	6,5	10,0	15,1	27,4
11,0	5,9	9,0	13,8	25,3
11,5	5,3	8,3	12,6	23,3
12,0	4,9	7,5	11,5	21,4
12,5	4,4	6,8	10,5	19,7
13,0	4,0	6,2	9,5	18,1
13,5		5,6	8,6	16,5
14,0		5,0	7,8	15,1
14,5		4,5	7,1	13,7
15,0		4,0	6,4	12,6
15,5			5,8	11,4
16,0			5,2	10,4
16,5			4,7	9,4
17,0			4,2	8,5
17,5				7,7
18,0				6,9
18,5				6,2
19,0				5,6
19,5				4,9
20,0				4,5

Tono-Pen XL tonometer (picture 5) is an electronic tonometer of contact that displays directly the IOP value determined both by measurement and the average value of four consecutive measurements. The IOP measurement (picture 6) is made after achieving the surface anaesthesia of the cornea. After measurement, it calculates and displays the standard deviation corresponding to the respective determination.



Picture 5 Tonopen XL tonometer

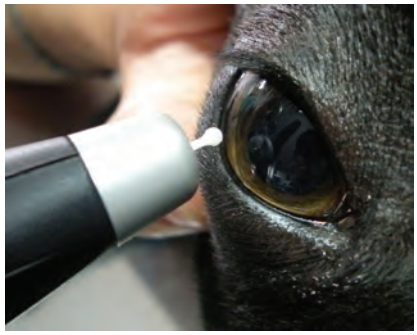


Picture 6 IOP measurement using tonopen XL tonometer

Tono Vet tonometer (picture7) is an electronic tonometer of contact, rebound type that displays directly the IOP value determined both by measurement and in the form of the average value of 6 consecutive measurements. Anaesthesia of the cornea is not needed for the

measurement of IOP. (Picture 8) It calculates and warns about the outrunning of the standard deviation corresponding to the respective determination. It doesn't need calibration and the measurement accuracy is of ±2 mm Hg.

Picture7 Tono-Vet tonometer



Picture 8 IOP measurement using Tono Vet tonometer

2. RESULTS AND DISCUSSIONS

If we analyze the factors that can influence tonometry, the best results, real values of IOP, are obtained when the animal is subjected to a minimal contention.

Each type of contention produces changes (increases or decreases of the IOP value) of the obtained result, depending on the way of accomplishment.

For instance, the manual, strong and incorrect contention of the animal's head modifies the facial aspect, produces pressure at the level of the ocular ball and thus, increased IOP values will be obtained. The main features of the four types of tonometers were evaluated comparatively, as a result of IOP measurement at the studied animals. (table 3).

The used tonometers present advantages and disadvantages as it follows: Schiotz tonometer is reliable, economic (we don't need specific and unspecific consumables); it is easy to be kept up and disinfected.

The major disadvantages consist in the way we measure the IOP value: a strict positioning (picture 2) on the cornea curvature is needed. (As a consequence of this fact, there are big congruence errors directly proportional to the difference between the curvature radius of the tonometer contact part and the cornea curvature radius in the contact area.) The contact of the tonometer with cornea and its maintaining on the surface implies local anaesthesia or animal's tranquilization

Tabel 3

The comparison of the main features using the evaluation on a scale from 1 to10

Features under consideration	Tonometer type			
	Schiotz	Perkins Mk2	Tono-Pen XL	TonoVet
Portability	10	10	10	10
Preparation of the device prior to measurement	5	7	8	9
Animal`s contention	5	5	8	9
Local/general anaesthesia	5	5	5	10
Measurement speed	5	3	9	10
Measurement precision	4	10	7	9
Sterilization/disinfection	6	6	10	10
Consumables specific to the measurement	10	10	7	7
Consumables unspecific to the measurament	10	7	9	9
Evaluation of the standard deviation	2	1	9	8
Reliability	10	7	8	8
Results / display / interpretation	5	6	7	10
TOTAL	77	77	97	109

The major advantage of Perkins Mk2 tonometer is the great precision of determination that is of 0,2mHg. The disadvantages are represented by the use of local anaesthesia or tranquilization, strict positioning on the cornea (picture 4), specific consumables and the long measurement time.

At Tono-Pen XL tonometer, the positioning on the cornea surface is not very strict (picture 6) and it has the advantage of providing the average value of four consecutive measurements by calculating the standard deviation.

The major disadvantages are represented by the low precision, specific and unspecific consumables, the necessity of calibration procedures and local anaesthesia. (7, 10, 11)

The main advantages of Tono Vet Tonometer are that it doesn`t need local cornea anaesthesia, it has a high precision and a high measurement speed. (7, 10, 11)

The major disadvantages are represented by the necessity of specific consumables and positioning (picture 8). We considered both the

advantages and the limits of these tonometers and we achieved tables of evaluation on a scale from 1 to 10 (table 3).

3. CONCLUSIONS

3.1. Presently, the veterinary ophthalmology uses the following tonometer types: Schiotz, Perkins MK2, Tono-Pen XL and TonoVet.

3.2. The IOP values depend on the animal`s way of contention, the measurement technique and speed.

3.3. If we consider all the main studied features, TonoVet tonometer presents the major advantages in order to obtain a real IOP value.

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