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CONTENTS

THE PREVALENCE OF GIARDIOSIS IN ANIMALS AND HUMANS IN IASI COUNTY WITH THE ESTABLISHMENT OF ZOONOTIC RISK

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Abstract
*The study on the prevalence of Giardia sp. in bovines was performed by analysing the cases recorded at Dancu Research Station, the results showing that this is a protozoan, commonly found in bovine farms, being one of the main causes of diarrhoea in calves. The source of contamination with Giardia sp. is represented by drinking water, breast milk and environment. In the period 2017-2020, the cases of giardiosis diagnosed in pets at the Faculty of Veterinary Medicine were analysed; a coinfection was found in dogs in a proportion of 64% with yeast cells, 22% with Cryptosporidium sp., 6% with Isospora sp. and only in 3% of cases **Giardia sp.** has been reported as the only pathogen involved in the clinical picture. The study on the prevalence of Giardia sp. in humans during 2017-2020 was performed using data provided by the Praxis Laboratory. The conclusions demonstrate the presence of a high rate of giardiosis in pets (dogs, cats), in farm animals (bovine), and in humans, each representing a source of contamination of the environment and of the other categories. The results showed for both humans and animals that drinking water can be a major source of infection with Giardia sp., requiring the much more frequent and rigorous control of drinking water.*
***Keywords**: giardiasis, opportunistic parasite, coinfection*

Introduction

Giardia intestinalis, also called *Giardia lamblia* and *Giardia duodenalis*, is one of the most common intestinal parasites in the world, occurring in both industrialised and developing countries, with approximately 2.8 million new cases reported annually. Giardiosis is a widespread protozoosis worldwide, with the parasitic reservoir consisting of a large number of healthy carriers. It is a parasitosis found in humans and animals, although the genotype adapted to each species is quite distinct. The clinical features of acute giardiosis in humans are similar to cryptosporidiosis and include severe diarrhoea, abdominal cramps, nausea, and weight loss. These symptoms may persist for several weeks or progress to a chronic disease. The infection can be asymptomatic or subclinical. Giardiosis in cattle, goats and sheep can cause malabsorption of nutrients, which may lead to weight loss. Microscopic examination of stool samples remains the basis of diagnosis for these parasites, although molecular methods and immunological tests can effectively replace microscopic approaches.

In 2017, 19.437 cases of confirmed giardiosis were reported in the EU/EEA. The EU/EEA notification rate was 5.5 cases per 100,000 inhabitants. The highest number of confirmed cases was reported in the United Kingdom, followed by Germany. These two countries accounted for 44% of all confirmed cases of giardiosis in the EU/EEA. Most of reported cases of giardiosis (60.1%) had been taken from the domestic market, except for three Northern countries (Iceland, Norway and Sweden), where 71% -83% of cases were associated with travel. Although the EU/EEA notification rate was stable over the period 2013-2017, the annual number of cases constantly increased. Giardiosis is the most commonly reported of the five parasitic diseases with possible contamination in food and water under mandatory EU surveillance. Giardiosis surveillance covers the entire population of most EU/EEA countries. However, a review in 19 Eastern European countries

showed discrepancies between the notification rates provided in the study and the rates officially reported in TESSy, suggesting under-reporting in all Eastern Europe. A quarter of EU Member States do not have surveillance systems for giardiosis and do not report cases. Giardiosis is common in dogs and cats in Europe and the United States, affecting animals of all ages, with a higher prevalence in young animals from weaning to 2 years of age. According to several epidemiological studies, it can be found in about 10% of faecal examinations in carnivores which have diarrhoea and are taken to the vet for examination. Epidemiological studies performed on the dogs used for breeding indicate that the parasite is present in almost 100% of cases, and that the prevalence of infection in dogs is up to 50%. These figures are identical or slightly higher than in helminth infections, making *G. duodenalis* one of the most common intestinal parasites in domestic carnivores. Giardiosis is particularly relevant in cattle, where infections associated with severe symptoms are commonly seen in young animals. Among the sources of zoonotic infection, cows are considered a major contributor, because the species and genotypes found in humans have also been isolated from cattle. In general, the infectious stages of *Giardia sp.* are excreted in the faeces of infected hosts and are able to infect susceptible hosts after ingestion. Humans can get the infection directly from contact with infected people (anthroponotic transmission), or animals (zoonotic transmission), or indirectly from contaminated food or water sources.

Material and method

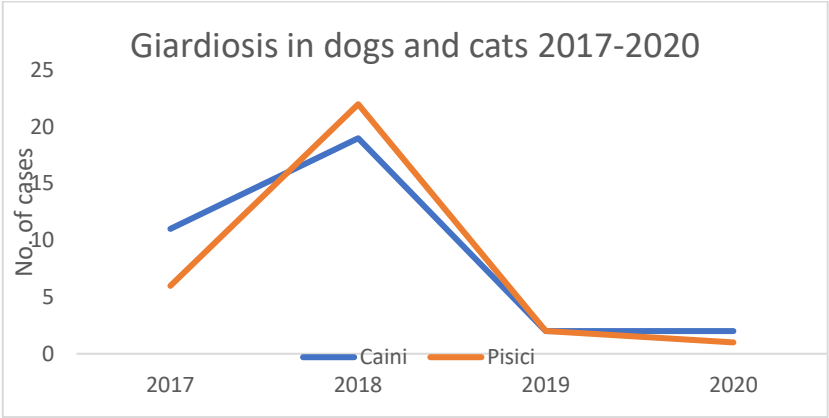
For the diagnosis of giardiosis, we used a direct microscopic examination with Lügol solution, the method of enrichment with saturated sugar solution, and quick test for the detection of Giardia Kit Antigen. The protozoan *Giardia duodenalis* can occur in two forms: trophozoite (vegetative form) and cyst. The vegetative form is found exceptionally only in watery stools, in the acute stage of the disease, especially in young women. The trophozoite is piriform, 15 µm long/6-10 µm wide. It has a body with bilateral symmetry with 2 nuclei, 4 pairs of blepharoplasts and 4 pairs of flagella, an adhesive disc and an axostyle. The cyst is oval and is 9-13 µm long/6µm wide. It has a thin, double outer membrane, 4 nuclei, and a bundle of flagella. The nuclei have a variable arrangement, often grouped at one end of the cell. In the axis of the cyst, we can see S-shaped flagellated remains, with sinuous disposition. In the native preparation made in Lugol solution, the cysts are obvious, brown-coloured.

The samples were analysed in the Paraclinical Laboratory of Parasitology, within the Faculty of Veterinary Medicine, and they were obtained from cattle, dogs and cats. Faecal samples were collected from the patients who came to the clinic for a diagnosis, with an expressed clinical picture, and we could work on fresh samples. The data analysis was performed for the period 2017-2020. We collected samples from cattle from the Research and Development Centre for cattle breeding in Dancu; they were identified with a unique code, and kept in vacuum bags, and we processed them in stages. In order to establish the prevalence of the protozoan *Giardia sp.*, the samples were collected only from animals with a digestive clinical picture - soft stool to profuse diarrhoea. The samples were collected only from calves with diarrheal syndrome, because adults can be asymptomatic carriers, in which case no treatment is recommended. The samples were collected over a period of 3 months.

In addition, we did an analysis of the prevalence of giardiosis in humans, the data being provided by Praxis Medical Test Laboratory in Iași, for the period 2017-2020.

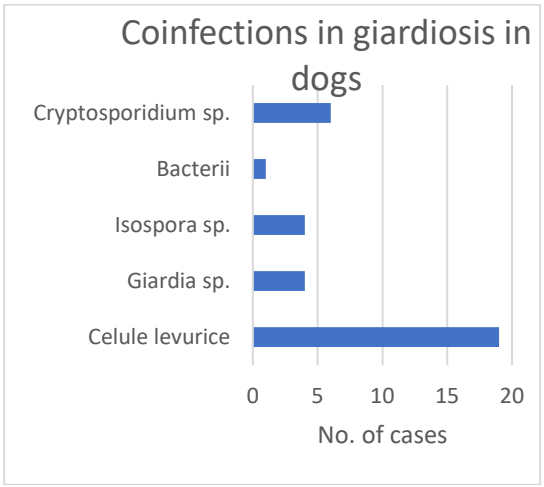
Results and discussions

The results obtained in the Paraclinical Laboratory of Parasitology within the Faculty of Veterinary Medicine

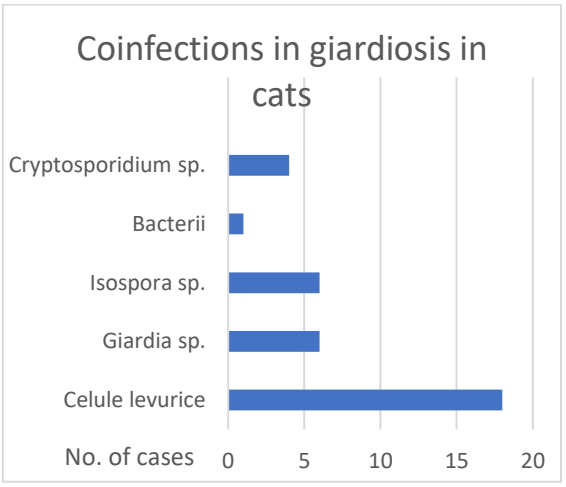


Graph 1. Giardiosis in dogs and cats 2017-2020

In the period 2017-2020, at the Faculty of Veterinary Medicine, 48 cases were recorded in pets, of which 25 cases in dogs and 23 cases in cats. In 2019, most cases of giardiosis were diagnosed, 15 in dogs and 8 in cats. Giardiosis is a protozoosis underdiagnosed in both veterinary medicine and human medicine, due to the sensitivity in making a correct diagnosis. The quick antigen detection test is expensive to use in every clinical case suspected of parasitosis, given the non-specific clinical picture. The diagnosed cases were due to the lack of a preliminary diagnosis, or due to the ineffectiveness of the treatment established subsequently. Many *Giardia sp. i*nfestations were treated without a definite diagnosis.



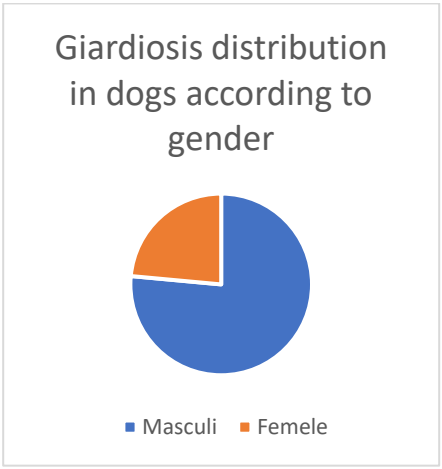
Graph 2 Coinfection in giardiosis in dogs



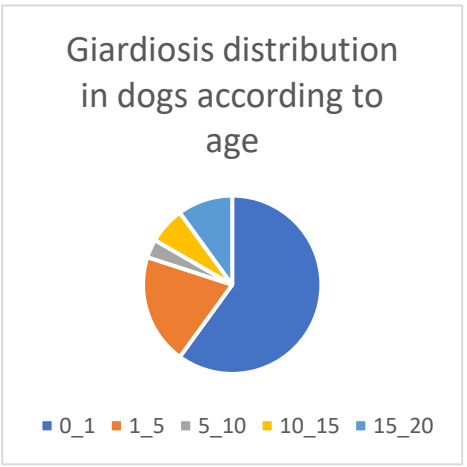
Graph 3 Coinfection in giardiosis in cats

In the cases diagnosed at the Faculty of Veterinary Medicine, a coinfection was found in dogs in a proportion of 65% with yeast cells, 22% with *Cryptosporidium sp.*, 8% with *Isospora sp.* and only in 5% of the cases *Giardia sp***.** was reported as the only pathogen involved in the clinical picture (graph 2).

In cats, a coinfection was reported in a proportion of 62% with yeast cells, 32% in coinfection with *Cryptosporidium sp.*, 9% with *Isospora sp.* and 14% in **Giardia sp.** was found to be the only pathogen responsible for the clinical picture expressed (Graph 3).

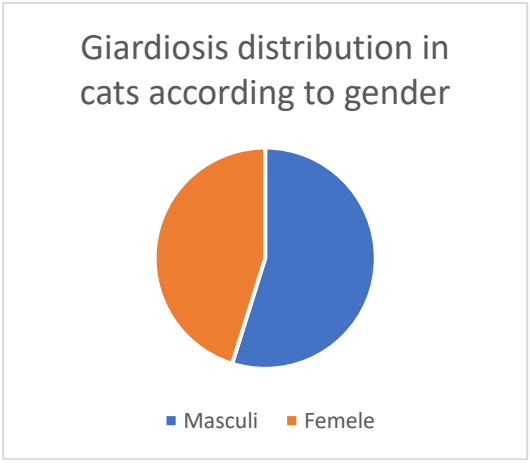


Graph 4 Giardiosis according to gender

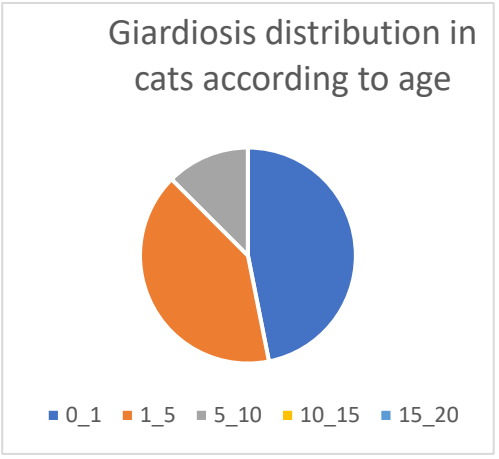


Graph 5 Giardiosis in dogs according to age

Of the total number of cases, 74% of cases were diagnosed in males and only 22% of cases were diagnosed in females. Taking into account that most cases were recorded in young dogs aged up to one year, uncastrated, we cannot blame the hormonal status, which can be a cause of lower immunity (graph 4). Of the total number of cases, 50% were recorded in young animals aged up to 1 year, 12% in the age category of 10-15 years, and 14% in the age category of 15-20 years, and only 7% in the age category of 1-5 years. The data correspond to those recorded in the literature, where giardiasis appears as a disease of the youth, with developing immune system (graph 5).



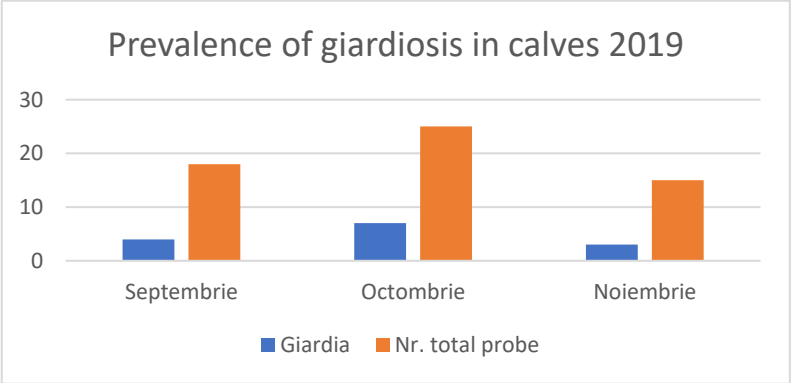
Graph 6. Giardiosis cats according to gender



Graph 7. Giardiosis in cats according to age

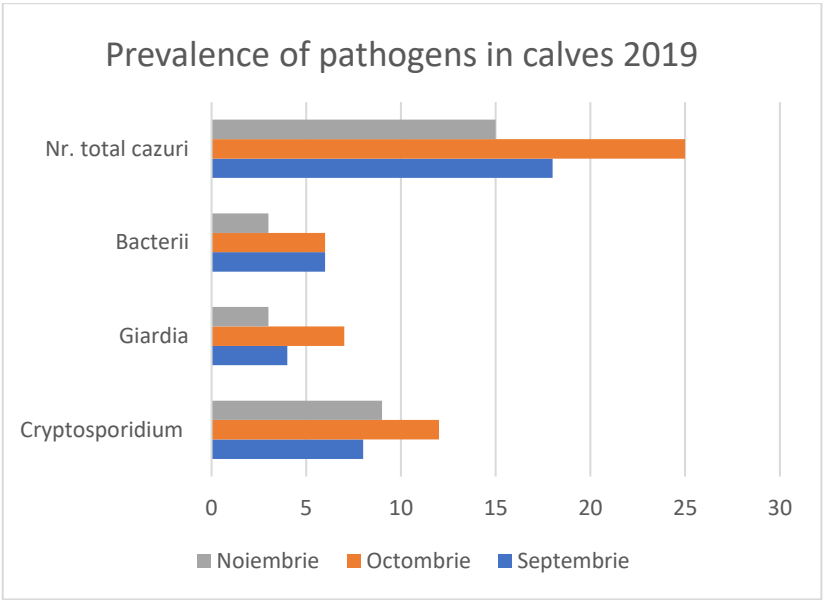
The distribution according to gender of the cases of giardiosis in cats, in contrast to the situation reported in dogs, is balanced by 55% of cases recorded in males and 45% of cases recorded in females (Graph 6). In cats, out of a total of 32 cases, 49% were diagnosed in cats aged 0-12 months, 41% in cats aged 1-5 years, and 13% in cats aged 5 to 10 years (Graph 7).

Scientific results: Cattle Research and Development Center in Dancu



Graph 8. Prevalence of giardiasis in calves 2019

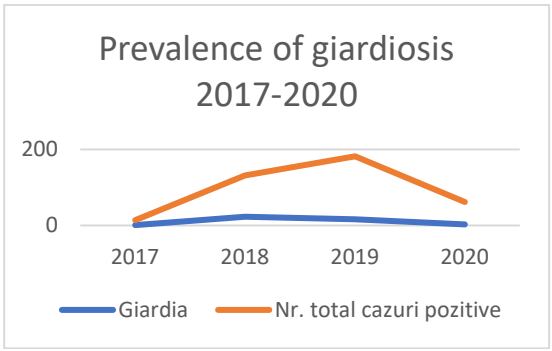
The samples were collected over a period of three months: 18 samples in September, 26 samples in October and 14 samples in November. From the total samples collected, *Giardia sp.* had a prevalence of 23% in the samples collected in September, 27% in October and 20% in November (graph 8).



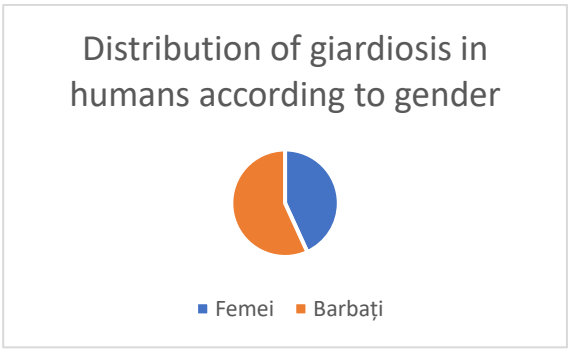
Graph 9. Prevalence of pathogen in calves 2019

Of the total number of samples collected in September, a prevalence of 43% of *Cryptosporidium sp. C*ysts was observed, 23% of *Giardia sp. c*ysts, and 33% of bacteria, pathogens causing diarrheal syndrome in calves up to the weaning period. In October the prevalence was 47% for *Cryptosporidium sp*., 29% for *Giardia sp., a*nd 24% for bacteria (*Clostridium*, *Camphylobacter*, *Salmonella*). In November, the prevalence was 61% for *Cryptosporidium sp.*, 19% for *Giardia sp.*, and 20% for bacteria (graph 9).

Results of the research conducted at Praxis Test Laboratory

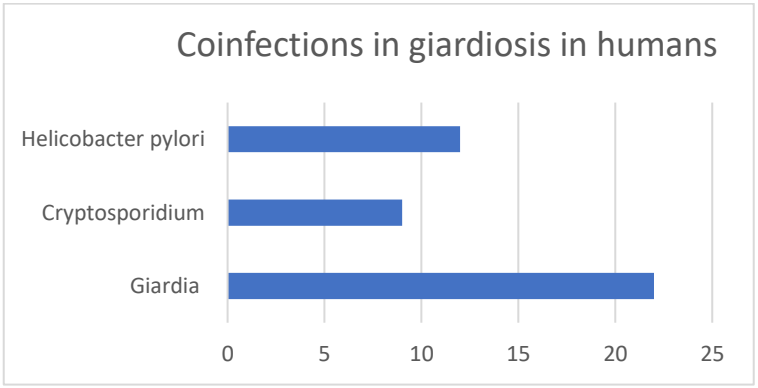


Graph 10 Prevalence of giardiosis 2017-2020



Graph 11 Distribution of giardiosis according to gender

In the period 2017-2020, 11.002 coproprasitological examinations were performed at Praxis laboratory, of which only 391 were positive. Of the total positive cases, 12% are represented by infection with *Giardia sp*. , and the remaining 88% are as follows: *Chilomastix mesnili, Iodamoeba buetschlii, Entamoeba hartmani, Entamoeba coli, Endolimax nana, Blastocystis hominis, Ascaris lumbricoides, Enterobius vermicularis, Cryptosporidium sp* (graph 10*).*
Regarding the distribution of giardiasis according to gender, it is balanced with 59% of cases in men, and 41% in women (graph 11).



Graph 12 Coinfection in giardiasis in humans

In 52% of cases, *Giardia sp.* was diagnosed as the only cause in the appearance of the clinical picture., which shows that it is an opportunistic parasite, but whose pathogenicity can produce a digestive picture. In 27% of cases, *Giardia sp. w*as diagnosed in coinfection with *Cryptosporidium sp., a*nd in 27% of cases in coinfection with *Helicobacter pylori,* which by local action modifies local biochemistry.

Conclusions

1.The research was conducted at the Faculty of Veterinary Medicine in Iași, Dancu Cattle Research and Development Center, and Praxis Test Laboratory in Iași for human medical tests, in order to establish the zoonotic risk of giardiasis and the main source of contamination with cysts of *Giardia sp.*

2.The results show that giardiosis is an underdiagnosed protozoosis in both veterinary medicine and human medicine, due to the sensitivity in making a correct diagnosis. The quick antigen detection test is expensive to use in every clinical case suspected of parasitosis, given the non-specific clinical picture. The diagnosed cases were due to the lack of a preliminary diagnosis, or due to the ineffectiveness of the treatment subsequently established. Many *Giardia* sp. infestations have been treated without a prior definite diagnosis.

3.The results obtained at Dancu Farm show that giardiasis is a protozoosis, commonly found in cattle farms, being one of the main causes of diarrhea in calves. The sources of contamination with *Giardia sp.* are represented by drinking water, cow milk and the environment. Taking into account the early age of the calves from which samples were taken, milk is the main source of cyst contamination, which means that the number of asymptomatic carrier adult cows is high within the farm.

4.The conclusions demonstrate the presence of a high rate of giardiosis in pets (dogs, cats), in farm animals (cattle), and in humans; each representing a source of contamination of the environment, as well as of the other categories.

5.Thus giardiosis is an opportunistic parasite, which we can contract from the environment, food or drinking water, the cysts of *Giardia sp*., being resistant to common decontamination methods.

6.The results showed for both humans and animals that water can be a major source of *Giardia sp*. infection, imposing the more frequent rigorous control of drinking water (in Romania water testing does not involve checking for the presence of *Giardia sp.* and *Cryptosporidium sp.* protozoa).

7.Cow milk is an important source of contamination with *Giardia sp.*, emphasising the importance of heat treatment before consumption.

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DIAGNOSTIC METHODS AND THERAPEUTIC OPTIONS IN DOG SKIN ALLERGIES - A SHORT REVIEW

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Abstract
Allergy is an exaggerated reaction of the body, which occurs after the contact with various substances that the immune system considers foreign and acts against them. Like humans, dogs can be allergic to various substances which, when inhaled or absorbed through the skin, airways or gastrointestinal tract, stimulate the immune system by releasing histamine that induces inflammation, erythema, edema and itching. It is considered that all the cases of allergies, whether low, moderate or severe, are genetic in nature. Dogs that become allergic to drugs, vaccines, food, dust, pollen, fleas, various household substances, etc., are "genetically programmed" to have an immune system capable of producing an allergic reaction. The allergic reaction is not due to medication, biological products, food or the environment, but it is considered to be a genetic trait inherited from parents. Because there are different types of allergies and many conditions that can cause skin problems in dogs, the symptoms they present differ from one allergy to another, from one patient to another, requiring both diagnosis and treatment to be made differential for each type of allergy and for each individual. The symptoms of allergic reactions can be diagnosed through allergic tests (intradermal or blood tests), the individual removal of allergens from the dog's living environment (in case of parasitic allergy), or by reagent therapy. Fighting allergies involves the use of antihistamines, anti-inflammatory corticosteroids, topical antipruritic substances, leukotrienes inhibitors, as well as various plant extracts with antiallergic effect.
Keywords*: allergens, allergic reaction, antihistamines, corticosteroids*

Introduction

Allergy is defined as a hypersensitivity or abnormal reactivity of the immune system to a particular substance. A large number of substances can act as allergens, most of which are represented by various drugs, insect proteins (flea saliva or mosquito bites), animal or vegetable proteins (food, pollen, mold spores, dust particles), immunological factors but also chemicals (for household use) that can cause allergies.

Most often the contact with the allergen causes local or systemic inflammatory reactions that determine the clinical symptoms of the allergy. The organs most commonly affected are the ones representing the entrance for the allergen (the so-called target organs: skin and mucous membranes, respiratory tract and digestive tract). Symptoms can be local or systemic, of varying intensity and severity, sometimes even lethal. Systemic reactions also occur when allergens are introduced directly into the bloodstream (through injections, infusions, transfusions, insect bites, etc.).

Allergies are quite common in dogs, regardless of breed, and the most common symptom associated with allergies is itchy skin, either localized (in a certain area) or generalized (all over the body) (*Bensignor E, 2013, Bruet V et al., 2012*). In some cases, the symptoms involve the respiratory system with manifestations of coughing, sneezing, shortness of breath or in other cases, allergic symptoms may begin with digestive disorders, manifested by vomiting and diarrhea.

Many experts believe that some allergies in animals are inherited *(Eichenfield LE et al., 2014; Merryman-Simpson AE et al., 2008; Sander I, et al, 2015)*. An inherited allergy is atopy or allergies to

pollen and plants. It is believed that certain breeds of dogs, both males and females are genetically predisposed to become sensitive to environmental allergens *(Marsella R. et al., 2012)*. Among the breeds prone to the development of allergies we can list: American Hairless Terrier, American Pit Bull Terrier, Bichon Frise, Bohemian Terrier, Boxer, Brussels Griffon, Bull Terrier, Bulldog, Chinese Crested, Dalmatian, English Cocker Spaniel, German Shepherd, Golden Retriever, Irish Setter, Labrador Retriever, Lhasa Apso, Maltese, Miniature Schnauzer, Pekingese, Poodle, Pug, Shar-pei, Shih Tzu, Scottish Terriers. However, any dog of any breed (or mixed-breed) can be allergic, and the age at which the allergy occurs for the first time is generally between 6 months and 3 years. Clinical signs may appear seasonally, but can sometimes be seen throughout the year. Itching is the most typical sign, and the face, ears, front legs, armpits, abdomen and tail base are the most commonly affected areas, but the scratches caused by intense itching can be found all over the body. Scratching can lead to secondary signs of wounds, scabs, skin and ear infections, hair loss and scaling. The skin is the main target of atopic dermatitis, but about 15% of the affected dogs also develop nasal inflammation (rhinitis) and asthma. Long-term or recurrent ear infections may be the only sign in a small number of dogs.

Although initially they appear to be harmless, skin allergies present a real risk of subsequent infection, which can lead to complications, because as the itching occurs, the animal scratches, bites or licks its skin frequently, leading to its sensitization and the appearance of local irritations, being more prone to infection with different bacteria *(Constantin C., et al., 2009)*. The essential role of the skin for the dog's health is already well known, it has different functions such as defense against harmful environmental factors, thermoregulatory role to maintain a constant body temperature, the role of ensuring cognitive functions that together with the fur, sebaceous and sweat glands helps the dog to integrate into the environment and also to influence its sexual behavior, but the skin also has a mitigating role in case of trauma due to its elasticity (*Miller WH., et al., 2013*).

Thus, it is important to know the causes that can lead to skin diseases, the general signs that indicate a dermatological allergy, the diagnostic methods and the most effective treatment depending on the type of allergy.

Allergies classification

There are different types of allergies, each with its own symptoms, classified according to the type of allergen and the areas of the body it affects.

Allergic reactions or hypersensitivity reactions (immune mediated) are classified according to their type:

Type 1 or immediate type (also called acute anaphylaxis), is common in dogs and can be caused by various proteins. This category includes allergies induced by insect bites, food allergies, various medications, allergic rhinitis and it usually begins with edema of the head and ears, urticaria, anaphylaxis and even death. Allergic shock, also called anaphylactic shock, is the most dangerous manifestation of an allergic reaction of this type, because it can cause death.

Type 2 or cytotoxic induces reactions like immune-mediated hemolytic anemia and immune-mediated thrombocytopenia, being rare and caused by drugs.

Type 3 or the immune complex is triggered by antibodies that come in contact with various substances which dissolve in the blood and form aggregates on the vascular walls. Type III allergies can trigger an inflammatory reaction, manifested by rash and itching. One can also observe an ischemic cutaneous vasculopathy that can appear after an injectable administration of some drugs (vaccines, antibiotics, etc.) but which has an affinity for additional places compared to the injection site.

Type 4 or delayed type hypersensitivity is represented by cell-mediated reactions with a delayed reaction with an average time of 2 weeks and which includes erythema and contact dermatitis as clinical signs.

There are various other ways to classify allergies, and some of them include: allergy to insects (fleas) or food allergy caused by a certain type of allergen, with clinical signs of allergic dermatitis manifested by urticaria, pruritus, irritation; inhalation allergy or allergy induced by the skin contact with the passage of the allergen in the body, manifested by dermatitis or allergic bronchitis; immunological factors that induce type I hypersensitivity which may lead to anaphylaxis or shock and delayed type hypersensitivity, depending on the time required for the immune response; genetic factors (in inherited forms) with manifestations of atopy or seasonal allergies.

Another classification of skin allergies, depending on the substance causing the reaction and the symptoms it causes, is: food allergy - caused by substances in certain foods: meat, eggs, dairy, fruit; drug allergy - when the body reacts to certain pharmaceuticals; allergy to insect bites - occurs as a result of insect bites - such as fleas, mosquitoes, bees or wasps; contact allergy - as a reaction after the contact between the skin and an allergen, such as household cleaners, disinfectants, pollen, feathers, various plants.

Flea bite allergy can be easily diagnosed because, in most cases, both fleas and their dirt can be seen on the animal's fur. Most dogs experience minor local irritation (especially at the base of the tail) due to flea bites, but in sensitive dogs an allergy to insect bites can induce an exaggerated inflammatory response, with flea saliva being one of the most commonly incriminated allergens in this pathology, causing flea allergic dermatitis. In the latter, the bite of a single flea can induce a severe local reaction with intense itching, which will cause the dog to bite and scratch, leading to the removal of a large amount of hair from the affected area *(Foster A, Foil C., 2003*). In areas with damaged skin there is a risk of developing a secondary bacterial infection.

Food allergy, also called food hypersensitivity can occur in almost any protein or carbohydrate component in a dog's diet (*Gaschen FP. et al., 2011; Carrión, P. A et al., 2014*). Food allergies can be caused either by predisposing factors, represented by the genetic disposition of some dog breeds such as Geman Shepherd, Cocker, Golden Retriever, Labrador, Bichon, Peckinese, Bulldog, or by triggers, represented by meat or dairy proteins, but also certain substances in spices and additives present in dog food. Allergic responses to dairy proteins, cereal gluten, chocolate, egg proteins, beef, pork, chicken, lamb or soy-containing foods were most commonly observed (*Pucheu-Haston CM., 2016*). All of these have been commonly associated with food allergies in dogs. Allergic phenomena given by certain foods can occur at any age and can produce mild clinical signs to intense clinical signs, especially when associated with atopy, which makes the diagnosis difficult to establish (*Craig J. M., 2016; Chandler, M. L., 2013*).

Allergy induced by various inhalants in dogs is often used as a synonym for atopy. The main inhalant allergens are pollen (plants - ragweed; trees - linden, oak, cedar, ash; grass), mold and dust mites. Many of these allergies occur at certain times of the year (seasonally during the flowering of ragweed, trees and grass), and allergies to mold and dust mites occur throughout the year (*Guaguère E, Prélaud P., 2006*). In humans, allergies that occur after inhaling allergens are generally manifested by upper respiratory signs such as sneezing, runny nose and eyes, coughing, redness of the face, etc. In dogs, inhalant allergy is manifested by itchy skin and very rarely by allergic rhinitis or bronchitis. Most dogs that manifest inhalation allergies begin to show signs between one and three years old. Affected dogs will often react to more allergens and will often associate flea or food allergies.

Contact allergy (contact dermatitis) occurs quite rarely in dogs and it results from direct contact with allergens, such as household substances, pyrethrins, pesticides used on lawn, various plants, materials such as wool or synthetic materials used in carpets or bedding, etc. Contact dermatitis can be of two types: *irritating*, which occurs from the first exposure to various allergens (detergents, soaps, cosmetics, solvents, acids, plants) and leads to skin lesions that appear immediately after contact or a few hours later; *allergic* which is a manifestation of a delayed hypersensitivity reaction, type IV, which intervenes when repeated contact with a substance that has produced sensitization occurs (metals, plastic, various plants, local anesthetics). In this last type of dermatitis, the immune system is involved with the formation of Memory T lymphocytes, and the symptoms appear hours or days after re-exposure (*Gedon Natalie et al. 2018)*.

Contact allergies can develop in virtually any dog breed and at any age and will manifest as skin irritation, redness, swelling, thickening of the skin and itching at the site of contact, but along with the removal of the allergen (when it has been identified), the dogs will recover, without the need for a specific therapy. Because most of these allergens come from the environment, their identification is difficult to achieve and it is possible for recurrences to occur after a certain period, by permanently exposing the dog to the allergen. If the allergen or allergens have been identified by intradermal skin tests or blood tests, the dog should be protected as much as possible from exposure to them.

Exposure to allergens over a long period of time sensitizes the immune system, and subsequent exposure to the same allergen or a related allergen causes an excessive reaction. Normally, the immune system protects against infections and diseases, but in the case of allergens the immune response triggers an exaggerated reaction that can be harmful to the body. The immune reactions involved in allergies are quite complex (*Reedy L.M. et al., 1997*). Most reactions involve allergenic protein molecules that combine with antibodies in the blood and then attach to mast cells, which are found in various tissues in the body. When the antigen and antibody react with mast cells, they release histamine, which induces local inflammation manifested by redness, edema and pruritus. It often happens that antibodies act in case of an allergic reaction to the saliva of insects that bite the animal, inhaled dust, pollen, food proteins, cleaning products, etc. When white blood cells (or leukocytes - the cells of the immune system that protect the body from foreign bodies) consider the above substances to be dangerous, they do not hesitate to attack in order to protect the body.

Genetic factors have been described in some papers as having led to the development of allergies, this being a predisposition inherited by some dog breeds from parents with a genetic background to atopy. Thus it is considered that the allergy is an acquired reaction of the immune system, which is genetically programmed and transmitted from generation to generation to several breeds. Although the pathogenesis of a genetic background is not fully understood, there is various evidence showing genetic abnormalities, a modified immune system that causes skin inflammation, and a skin barrier defect (*Bizikova P., et al., 2015*; Morar N. et al., 2006).

The prevalence of atopic dermatitis is estimated today worldwide at about 25% of all diseases encountered in dogs. Although the pathogenesis of allergies is not fully known, in the case of such a condition in a dog, a laborious diagnosis is necessary to establish an appropriate treatment for the type of allergy.

Diagnostic methods of skin allergies in dogs

An accurate diagnosis of the causes of a skin disease requires a detailed history, physical examination and appropriate diagnostic tests. Many skin diseases have similar signs and an

immediate diagnosis may not be possible. Different laboratory procedures can be established based on the dog's history and physical examination. These may include microscopic analysis of skin and hair scratches, hair cultures or skin swabs, specialized skin tests, blood and urine tests, and even biopsies. It may take several days for laboratory results to be available. For an accurate diagnosis, several examinations and evaluation of the response to the applied treatment are often necessary.

The diagnosis of skin allergies is sometimes difficult to make, so a differential diagnosis is needed depending on location, age of onset, breed, clinical signs and history of the disease. Thus, hypersensitivity to ectoparasites (given by flea bites) must be differentiated from food hypersensitivity (given by meat proteins and certain constituents in food) and atopic dermatitis (given by environmental allergens). Dog allergy testing cannot diagnose the type of allergy, but it is useful to identify the allergen or allergens and to institute a specific treatment (*Hill PB, et al., 2006; Miller WH et al., 2013*).

The main sign of an allergy is skin irritation, manifested by intense itching, bites on the legs, skin lesions that appear through scratches, agitation and anxiety.

Flea dermatitis is an exaggerated allergic reaction of the immune system after the contact with certain components of the flea saliva. It is the most common allergy found in dogs and it is a disease that produces severe itching (pruritic disease) and predisposes to the development of secondary skin infections. The diagnosis of flea allergy is made based on the symptoms, the antecedents, the clinical signs (frequently tail lesions) and the positive reaction to the flea control methods (*Bruet V et al., 2012*). The diagnosis of allergic dermatitis in flea bites is also based on the highlighting of fleas or flea feces.

Reactions to food intolerance in humans and animals are variable, usually dose-dependent, and can occur at any age (*Valenta R, et al., 2015*). Food allergy includes immunologically mediated reactions to food proteins, in which numerous immune cells and molecular mediators participate.

Signs may appear at any time, sometimes a few hours or days after consuming the incriminated food product and can last for hours or days. The differential diagnosis of food intolerance is broad (*Carrión, P. A., Thompson, L., 2014*), there are no specific diagnostic tests, and identifying the culprit foods can be a challenge, as several food groups may be involved in the same individual. Objective testing of food intolerance requires a double-blind, placebo-controlled food challenge, but it is rarely used (*Craig J. M., 2016*).

The International Committee on Allergic Diseases of Animals has developed a set of practical guidelines that can be used to help diagnose atopic dermatitis as effectively as possible *(Olivry T et al., 2013*). These sets provide an overview of the diagnosis of atopic dermatitis and involve three distinct approaches, such as: eliminating other skin conditions that overlap with atopic dermatitis, with similar clinical signs; evaluation and detailed interpretation of the clinical history of skin diseases; evaluation of skin reactivity by intracutaneous testing or by serological determination of allergen-specific IgE *(Ciprandi G., 2017).*

The diagnosis of allergies is often based on demonstrating a correlation between sensitization to a particular allergen, the production of specific IgE and the history of symptoms that occur after the exposure to that allergen. In patients with suspected IgE-mediated reactions or conditions, the diagnostic algorithm should include clinical evaluation and molecular allergen sensitization evaluation tests (*Ciprandi G., 2017; Harvey N. D. et al., 2019*).

In human medicine, there is used in the case of allergies the molecular diagnosis, which has been improved in recent years due to the production of standardized test material. Molecular diagnosis of allergies already provides valuable information on patients' individual sensitivities,

which, when combined with history and objective examination, may be relevant to clinical decision making. Molecular diagnosis contributes to the identification of different phenotypes of allergic populations and supplements tests for the determination of specific serum IgE in allergen extracts (*Ciprandi G., 2017*). Conventional tests, which use whole proteins, have the advantage of including most relevant allergens, which are mainly stable and found in large quantities in allergen sources, and are also useful if the relevant molecular allergens are not yet commercially available.

For a correct diagnosis of food allergies, it is necessary to evaluate both the positive or negative values of molecular allergens, along with the overview. Factors leading to the loss of oral tolerance and the onset of food allergy in some sensitized individuals are currently vaguely defined, but the loss of immunological tolerance is known to be based on several biological pathways, with inflammation mediated by T helper 2 (Th2) lymphocytes representing the key element in food allergy (FA). FA occurs as a result of the development of an abnormal immune response to food antigens and involves a first stage of allergen sensitization (*Hill P., 2002*). Sensitization may occur in the digestive tract, skin or may be clinically expressed as a cross-reaction in individuals previously sensitized to inhaled allergens. Skin epithelial cells, as well as those in the digestive tract, respond to tissue injury, inflammation or activation of the innate immune response, through the secretion of a network of cytokines that promote allergic inflammation. Recent data (*Hensel Patrick et al., 2015; Incorvaia C. et al 2015*) suggest that sensitization to trophallergens in the skin may be the central event in inability to obtain or the loss of oral tolerance. The loss of integrity of skin epithelial cells predisposes to sensitization. Skin sensitization appears to prevent the development of oral tolerance by inducing a Th2 response. This may explain the high prevalence of FA in dogs with atopic dermatitis. It has been observed that cutaneous dendritic cell (DC) subtypes are similar to the lamina propria DCs (*Incorvaia C. et al 2015*). By secreting interleukin-10 (IL-10), macrophages promote the regulatory response mediated by regulatory T lymphocytes (LT reg) (*Hensel Patrick et al., 2015; Incorvaia C. et al 2015*). LT have been assigned a number of roles in relation to modulating the immune response to food antigens. Intraepithelial lymphocytes appear to have a tolerogenic role, being involved in immune surveillance and in maintaining immune homeostasis.

Allergen sensitization, at the skin or digestive level, leads to the failure of the installation and maintenance of immune tolerance and this is the *primum movens* in the development of FA (*Outerbridge C., 2013*). The elucidation of the pathological mechanisms involved in the sensitization process is the starting point in identifying the factors that determine the immunological tolerance sustained naturally or induced by treatment.

The evaluation of a dog with allergic dermatitis requires a step-by-step evaluation for a more concrete diagnosis. A differential diagnosis is needed to highlight the factors that led to the disease (*Hensel Patrick et al., 2015*) (Table no 1).

Table 1

Important differential diagnoses for pruritic skin diseases in dogs
(After Hensel et al. BMC Veterinary Research)

Ectoparasitic skin diseases	**Fleas**
	Scabies (*Sarcoptes scabiei*)
	Demodicosis
	Cheyletiellosis
	Pediculosis

	Otoacariasis (*Otodectes cynotis*)
	Trombiculiasis
	Nasal mites (*Pneumonyssus caninum*)
Microbial skin infections	*Staphylococcal pyoderma*
	Malassezia dermatitis
Allergic skin diseases	Flea allergy dermatitis
	Atopic dermatitis
	Food intolerance/allergy
	Insect bite hypersensitivity
	Contact dermatitis
Neoplastic disease	Cutaneous lymphoma

The usual methods of diagnosing skin allergies are most often based on the skin areas where the signs appear, but these elements may not always indicate the type of allergy, so differentiation techniques are needed such as hair combing to highlight possible fleas, scraping of the damaged skin, tearing of hair from the injured area and cytological examination.

Depending on the situation, the clinician may consider certain steps, namely: in case of flea allergic dermatitis, skin lesions often give the first indication and are often located in the lumbosacral area to the base of the tail and on the inner or outer thighs (fig no. 1); food dermatitis induces pruritus in the facial area (muzzle and around the eyes), on the limbs which will cause intense licking and chewing of this area, pruritus in the anal area, recurrent ear infections, pruritus on the abdomen (fig. no. 2); skin dermatitis caused by fungi with erythematous and hyperpigmented lesions (fig. no. 3); atopic dermatitis with rashes in different regions of the body due to environmental or immunologically induced factors (fig. no. 4).

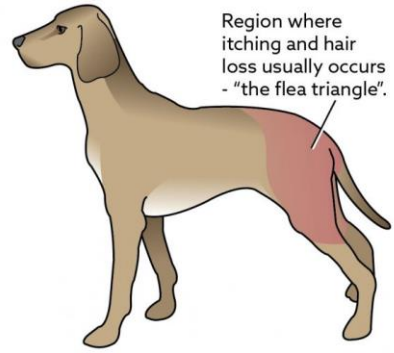

Fig. 1. Distribution of skin lesion and pruritus associated with flea allergy dermatitis (www.vcahospitals.com)

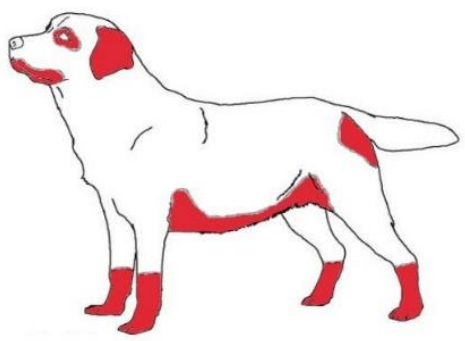

Fig. 2. Common distribution of clinical lesions and pruritus associated with canine atopic dermatitis and food allergy (www.molecarepetvets.com)

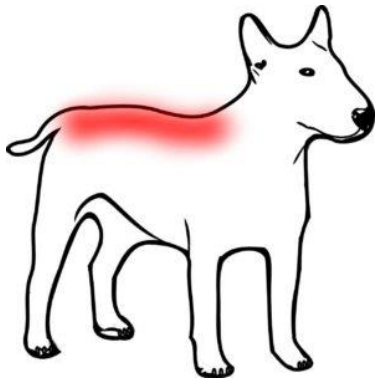

Fig. 3. Distribution of skin lesions and pruritus associated with hypersensitivity or contact allergy (www.walkervillevet.com.au)

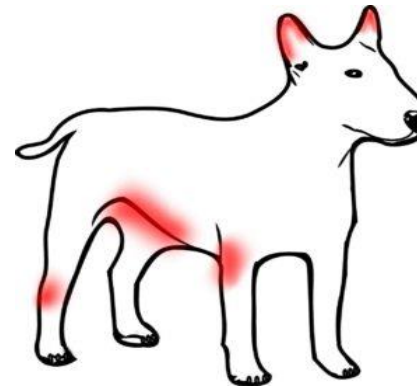

Fig. 4. Distribution of skin lesions and pruritus associated with mite infestation (www.walkervillevet.com.au)

In atopic dermatitis, the clinical overview is polymorphic, with intense itching, which makes the diagnosis to be established by corroborating the anamnestic data, age, the results of clinical and paraclinical examinations, as well as the results of allergic tests. Atopic dermatitis is located especially on the face, abdomen, limbs, axillary area and the generalized form occurs in a small percentage (25-30% of cases of atopic dermatitis) (*Hillier A. et al., 2001*). Skin lesions vary from case to case, with erythematous and papular dermatitis in the early stages of the disease, accompanied by scratching lesions, abrasions and scaly patches with alopecia. Subsequently, various manifestations of secondary pyoderma are observed, such as pustular impetigo, folliculitis or furunculosis (*Hill P., 2002; Schlotter YM., et al., 2011*). Progressively, the lesions common to chronic dermatitis are installed: alopecia, hyperkeratosis, hyperpigmentation, seborrhea. Frequently, along with atopic dermatitis, allergic dermatitis caused by flea bites is observed in dogs (with dorso-lumbar location and at the base of the tail), which explains the clinical manifestations of the two forms, leading to a difficulty in establishing the diagnosis.

As manifestations associated with atopic dermatitis found in a large percentage (about 50% of cases) there may be mentioned: bilateral otitis externa, conjunctivitis, chronic rhinitis. Dermatitis is initially seasonal, in 80% of cases starting in the warm season when the main allergens are seasonal pollen, but later the disease is persistent with manifestations throughout the year when allergens persist in the environment (dust mites). Atopic dermatitis usually begins at the age of 6 months to 5 years, certain predisposed breeds being terriers, Spanish cocker, Labrador, Golden retrivers, Shar peis, German Shepherd, but any dog breed may be affected.

The diagnosis of atopy will be made according to the symptoms, seasonality, response to drugs, excluding other similar allergies such as food or parasitic allergies, infections or fungal skin infections (*Guaguère E, Prélaud P., 2006*).

Cutaneous tests can be done by puncture or intradermally: the puncture test is done by stinging the skin on the dorso-lumbar area with several allergens, in a very small amount. The stung area will turn red if the dog is allergic. The intradermal test can only be used if the puncture test is not conclusive. In this case, the allergen is injected under the first skin layers.

Serological (blood) tests are used especially in patients with severe conditions (superinfections).

Therapeutic options for skin allergies of dogs

The therapy of dermatological diseases does not involve focusing only on the skin, it must be summarized mainly to eliminate the cause that led to the diagnosed condition. Allergies are most often diagnosed after specific tests have been performed.

A good management plan for skin allergies requires the use of different treatments, reasonable understanding and patience from the pet owner because the results can occur after long periods of treatment, frequent assessments of therapeutic progress so that the drug protocol can be adjusted accordingly as required.

The complexity of skin lesions due to intense pruritus, their multitude in different areas, their evolution towards healing or their aggravation towards superinfection but also the variety of medicinal solutions available today make the elaboration and implementation of the therapeutic protocol a challenge. Dermatological pathology is complex, frequent, multifactorial, with clinical manifestations ranging from simple clinical forms to severe forms, which makes the patient's care start from the rules of hygiene and proper skin hydration, the use of drugs with topical or systemic application, and to the use of current products, such as monoclonal antibodies. They are increasingly used because they prove their effectiveness in severe forms of the disease, becoming the medication of choice.

There are various treatment options needed to alleviate or inhibit allergic reactions to allergens (*Olivry, T. et al. 2015*).

Removing parasites from the animal (fleas) by external deworming with the multitude of specific substances existing for dogs but and from the environment in which the animal lives (cage, blanket, basket) by spraying with various specific substances is one of the first steps as a therapeutic option in parasitic dermatitis. The second step is to treat pruritus with corticosteroids and antihistamines for a certain period of time (about two weeks), and in case of bacterial or fungal complications, specific treatment is recommended both locally and systemically.

Food allergy requires primarily the identification of allergenic components (elements) in food, their elimination and the use of a hypoallergenic diet, this being the most balanced method of testing allergies given by the proteins of some foods. Because it takes at least eight weeks for all other foods to be eliminated from the body, the dog should receive a special diet exclusively for eight to twelve weeks until a positive response and clinical signs improve. Dietary dermatitis usually does not respond well to corticosteroids or other medical treatments.

Atopic dermatitis is a condition that the animal may face throughout life, so long-term management and regular veterinary examinations are necessary (*Shaw S., 2013*). Treatment involves a number of options such as avoiding allergens that cause the disease, itching control, improving hygiene and food, controlling parasitic factors, and immunotherapy (for example, an allergy vaccine).

The treatment of skin dermatitis depends largely on the duration of the specific season of allergies and may involve different methods of therapy (*Patel A, Forsythe P, 2008; Solcan Gh., et al., 2003*). Treatment options include therapy of allergic symptoms with topical and oral medication or allergy testing and desensitization injections to treat the leading cause of itching (*Scott DW, et al., 2001*).

Anti-inflammatory therapy with drugs with antiallergic and anti-inflammatory effect, such as corticosteroids (prednisolone-Prednicortone), or antihistamines (Histamine control, Chlorpheniramine, Diphenhydramine-useful and to relieve itching, Ceterizine, Loratidine, Clemastine, Hydroxyzine, Amitroptyline, Cyproheptadine), will quickly block the allergic reaction in most cases. The use of topical corticosteroids is done in order to relieve erythema, inflammation, pain and itching. Steroids used for injectable or oral administration, such as cortisone or

prednisone, act quickly and effectively to relieve pruritus, but their use should be done in the short term because they induce various side effects (increased thirst with frequent urination, insatiability which will lead to weight gain and obesity, increased blood pressure, even skin infections and hair loss). Also, topical steroid products such as hydrocortisone, betamethasone and triamcinolone should be used with caution for short periods of time, as overuse can predispose to skin infections due to the fact that they induce thinning and aging of the skin or create blackheads.

Supplementing the diet with fatty acids can improve the response to steroids and antihistamines in some cases. Omega 3 and 6 are essential fatty acids derived from various sources such as fish oil, flaxseed oil and vegetable oils. They have both a skin softening effect and an anti-inflammatory effect. They require administration for a long period of time, 1-3 months to observe a beneficial effect, fatty acids being ideal, combined with other specific products, for the treatment of allergic dermatitis in dogs, today being available on the market a wide range of products in various commercial forms (capsules, powder, liquid or chewable tablet). Fatty acids work synergistically with antihistamines to help reduce allergic skin inflammation and itching, but are not recommended in cases of dogs with other medical disorders, such as high cholesterol or clotting problems.

There are new alternatives to block specific chemical signs associated with cutaneous pruritus in dogs, which include daily oral medication such as oclacitinib (Apoquel), but also long-acting injections (Cytopoint, 30-day effect).

Symptomatic allergic treatment with mild or seasonal clinical signs can be managed by using topical medication such as shampoos, conditioners, sprays or with oral antihistamines, fatty acids (salmon oil), steroids and cyclosporine (Sporimune) (*Nuttall T., et al., 2014*). Hypoallergenic shampoo therapy can be soothing for the itching and inflammation of the skin. Antimicrobial shampoos (with chlorhexidine) are also used to help prevent scarring from becoming infected. Shampoos, conditioners and sprays used for allergies contain ingredients that help reduce itching, such as topical anesthetics, antihistamines, anti-inflammatories or plant extracts. For dogs with skin allergies, frequent baths are beneficial because they help reduce allergens that accumulate on the skin, but it is essential to use shampoos specially designed for pets due to pH differences. It is important that the shampoos are gentle so as not to induce dry skin and fur, so do not use tar or benzoyl peroxide-based shampoos.

The treatment of contact dermatitis primarily involves identifying the causative agent and stopping the contact with it, as well as avoiding further contact. In addition to pharmacological treatment, you can also use non-pharmacological treatment that aims to fight itching and moisturizes the skin. Bran colloid baths, warm showers, hot compresses and emollients applied after the bath can be used. Pharmacological treatment will be done with astringents (calamine), to reduce suppuration in the lesions and relieve itching.

Hyposensitization or desensitization therapy will be applied if specific antigens are identified by allergy testing and thus the patient may be injected with an allergic serum or anti-allergic vaccine. With this treatment, very small amounts of antigen are injected weekly in repeated doses, which aim to reprogram or desensitize the immune system. The effectiveness rate varies depending on the dog's response. Significant improvements in clinical signs were observed in approximately 50% of the treated dogs, and a reduction in pruritus was observed in approximately 25% of the dogs, which involved a decrease in the dose or in the frequency of corticosteroid use.

Conclusion

The treatment of atopic dermatitis is a complex and long-term one, which requires a diagnosis and a therapy adapted to each individual.

Animals with atopic dermatitis are prone to secondary skin infections, ear infections and fungal infections (Malassezia), which will lead to sensitive and damaged skin. For these reasons, flea control, diet control and special shampoo baths should be done regularly, and antibiotics and antifungal medications may be needed to treat any secondary infections.

The treatment of skin allergies will be differentiated depending on the type of allergen and will include external and internal antiparasitics, corticosteroids, antihistamines, antibiotics, antifungals, fatty acids essential, psychotropic, antiseborrheic, a balanced diet, hygiene of the animal but also of the area where it lives.

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RESPONSE TO POLIOVULATORY (POV) TREATMENT, BY ULTRASOUND IN SUFFOLK BREED

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Abstract
This study was done on a batch of Suffolk beef sheep, acclimatized in Romania.. The success of an ET protocol in sheep depends on many factors, but in the end, what matters is the number of embryos obtained. Embryo recovery (recovery rate), after poliovulation (POV), is an essential step in ET. The aim of our experiment was to observe the ovarian reaction (follicles –F, and corpora lutea-CL) to the treatment of Suffolk sheep polyovulation, The aim of our experiment was to observe the ovarian reaction to the treatment of Suffolk sheep POV. A number of 3 Suffolk sheep were poliovulated at the reproductive season, using P4-FSH-PGF protocol. The POV method was based on the administration of sponges with intravaginal progesterone 12 days, followed by 500 IU FSH: LHp in decreasing doses in the last 4 days, and a PGF on day 11. The poliovulatory ovarian response was monitored by transectal ultrasound, before estrus was detected, and on the day 7. The ovulatory response following POV treatment was assessed by CL counting. In two out of three sheep, CL was identified, despite the fact that they all had follicular growth, in sheep no. 2, no dehiscence occurred. The total number of formations observed was 26 CL. The distribution of CL between the two right and left ovaries, in the three cases examined was thus 8 CL on the right ovary and 7 CL on the left (in sheep 1), no CL in sheep 2 and 6/5 in sheep no. 3. The POV response to FSH in the Suffolk breed was an average of 8.6 F/sheep of the developed follicles. Our goal was in fact to follow the ovarian dynamics in these meat breeds, in order to apply the best treatment schemes, for successfully apply embryo transfer.
Keywords: *meat sheep, Poliovulation, MOET, embryo transfer*

Introduction

Small ruminant ET is a well described and yet underexploited animal breeding technology. The size of sheep, aspects of their anatomy and seasonal reproductive behaviour, present challenges not common to cattle. Those considerations have not deterred serious breeders and ET practitioners in sheep producing countries

Reproductive activity in sheep is characterized by a seasonality influenced by several factors such as photoperiod, latitude, temperature, nutrition and breed.

In sheep, some anatomical peculiarities limit the application of traditional reproductive biotechnology used in cattle.

Sheep estrus cycle has 17 days on average and can vary from 14 to 19 days. The estrous cycle is divided into a follicular phase for two to three days and luteal phase of 14 to 15 days. Small ruminants are reproductively seasonal species.

Biotechnology of Embryo Transfer is applied to females of superior genotic and aims to increase the frequency of their genes by increasing their progeny. ET allows the transference of embryos from superior females (donors) to matrices with low genetic value (recipients) or embryo freezing for later use.

Obtaining embryos is influenced by the development dynamics of the ovarian follicles, but also by their ovulation (Hafez ESE 2008).

Response to super-ovulation: approximately 25% of programmed donors will not respond to super-ovulation treatments. Some never respond, some may respond on a subsequent program. If the donor responds, she may produce from 1 to over 30 embryos, with average 8 to 12 depending on the breed, time of year, condition of animal (Ahmed and Derar 2015).

Our goal was in fact to follow the ovarian dynamics in these meat breeds, in order to apply the best treatment schemes, in order to successfully apply embryo transfer.

Materials and Methods

The present study was conducted during normal seasonal breed (autumn) in the N-Eastern region of Romania. This period is accepted as it represents the onset of the natural breeding season of smal ruminants, in this time the ovarian activity are vary intense.

Selection of the ewes was performed after breeding offspring, and after a complete clinical and gynecological examination.

Many combinations of treatments for the purposes of embryo collection and transfer are available. Estrus can be synchronized by the administration of progestagens such as progesterone

All groups received an intravaginal sponge (Chronogest, Intervet) containing 30 mg fluorogestone acetate (FGA) for 12 days. A number of 3 Suffolk sheep were poliovulated at the reproductive season, using P4-FSH-PGF protocol. The POV method was based on the administration of sponges with intravaginal progesterone 12 days, followed by 500 IU FSH: LHp in decreasing doses in the last 4 days, and a PGF on day 11.

Through ultrasound monitoring, the ovarian evolution can be observed in the dynamics. The endorectal technique is much more effective compared to the transabdominal one.

Result and discutions

In two out of three sheep, CL was identified, despite the fact that they all had follicular growth, in sheep no. 2, no dehiscence occurred. The total number of formations observed was 26 CL. The distribution of CL between the two right and left ovaries, in the three cases examined was thus 8 CL on the right ovary and 7 CL on the left (in sheep 1), no CL in sheep 2 and 6/5 in sheep no. 3. The POV response to FSH in the Suffolk breed was an average of 8.6 F/sheep of the developed follicles.

Many combinations of treatments for the purposes of embryo collection and transfer are available. Estrus can be synchronized by the administration of progestagens such as progesterone implants or synthetic progestins (flurogestone acetate, FGA; medroxyprogesterone acetate, MAP) given either orally or by the insertion of a vaginal sponge. The most widely used synchronization device for goats is the control internal drug release progesterone implant which is inserted in the goat’s vagina using a special applicator. Most traditional schemes consist of a long progestagen (12‑18 days) treatment; recent protocols use a shorter progestagen treatment (5‑9 days) accompanied by a prostaglandin F2 α analogue injection.

For the induction of superovulation of donor sheep, pituitary extracts of follicle-stimulating hormone (FSH) and pregnant mare serum gonadotropin (PMSG) are the gonadotropins most used. Commercially available FSH products are: Pluset .

Several protocols can be used for superovulating sheep, most commonly the injection of multiple doses of FSH on the last 3 to 4 days of the progestagen treatment. Due to the short half-life of the FSH molecule, it is traditionally administered every 12 hours. One example is the twice-a-day injection of a series of decreasing doses of FSH (5, 5; 3, 3; and 2, 2mg per injection), with a total dose of 20mg, with the next to last injection accompanied by progesterone removal and an injection of 150ug of a PGF2α analogue.

Fig 1. Ovarian follicle monitoring Transrectal ultrasound in sheep

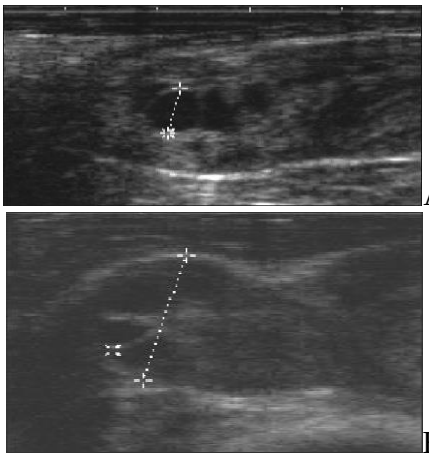

Fig 2. Evolutionary follicles from 3 to 9 mm, during poliovulation (A, B)

Fig 3. Monitoring multiple ovulation of the luteal body Transrectal ultrasound in sheep

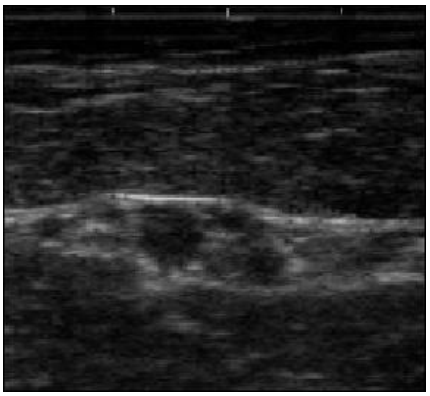

Fig 4. Identification of corpora lutea 6 days after their oestrus and counting

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NEURONAL POPULATION OF THE MESENCEPHALON, RHOMBENCEPHALON AND SPINAL CORD IN ZEBRAFISH *(DANIO RERIO)*

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Abstract
Anatomically central nervous system in zebra fish is represented by telencephalon, diencephalon, mesencephalon, mielencephalon and metencephalon. Histological study of neuronal population of mesencephalon, rombencephalon and spinal cord was made on 10 adult zebra fish, both sexes. Mesencephalon is the median vesicle of the brain having 3 main components: cerebral pedunculs, tegmentum and optic tectum. Tectum has seven layers of fibers and different celular composition. Rombencephalon comprise: cerebelum, ponce and medula oblongata and controls the autonomic functions and equilibrium. Cerebelum is composed by corpus cerebeli and valvula cerebeli, and together with ponce forms the metencephalon.Cerebelum have three layers of neurons: molecular, Purkinje cells and granular. Mielencephalon is represented by medulla oblongata, which is continuing caudally with the spinal cord, and is closing the 4th ventricle. Histologically, medula oblongata have small ovalar cells and small glial cells. In ventral spinal cord four domains are identified: p3, pMN, p1 and 0, composed from neurons having common features.
Key words: zebrafish *(Danio rerio), nervous system, histology*

Introduction

Danio rerio or zebrafish belongs to the family Cyprinidae, genus Danio, comes from India. The information provided by the development, genetics and sequencing research of the zebrafish genome allows their use as a model in various studies (Lieschke and Currie 2007). Genome sequencing, genetic changes that can be induced have led to the creation of numerous models of zebrafish for the study of human tumors, cardiovascular disease, Alzheimer's, Parkinson's, muscular dystrophies (Best and Alderton 2008; Guyon et al. 2007; Lieschke and Currie 2007; Zon and Peterson 2005). In addition, zebrafish has recently been used to assess drug toxicity (Eimon and Rubinstein 2009; McGrath and Li 2008, Strungaru et all., 2018).

The midbrain is the middle vesicle of the brain in developing vertebrates. It comprises three main components, as in other vertebrates, namely the cerebral peduncles, the tegmentum and the optic tectum. The optic tectum (OT) is a key center for processing sensory information that receives most of its afferents from the retina and builds an image of the physical environment. Through connections to multiple regions of the brain, it integrates visually acquired information with motor inputs and outputs to initiate appropriate behavioral responses. Tectum plays major roles in controlling eye movement and sensory-motor actions and has seven tangential layers. These layers have different fibers and cellular compositions.

The posterior brain or **rhombencephalon** includes the cerebellum, bridge and medulla oblongata and controls autonomic functions and equilibrium. The cerebellum consists of the corpus cerebelli and the cerebelli valve and together with the bridge forms the metencephalon. The myelencephalon is represented by the medulla oblongata, the caudal portion that continues with the spinal cord, closing the fourth ventricle and having the role of controlling involuntary vital functions. The terms metencephalon and myelencephalon are more significant in mammals and birds because in these vertebrates the metencephalon seems to be clearly separated from the myelencephalon, which is not the case in zebrafish. The spinal cord is formed from the rest of the neural tube.

Materials and methods

The study was conducted on 10 zebrafish, 5 males and 5 females. The samples taken were the fish entirely, initially placed for 4 hours in 4% formaldehyde. After this prefixation, they were sectioned mid-sagittally and transferred to the Bouin fixator for 48 hours. The pieces were dehydrated, clarified, embedded in paraffin, sectioned at 5µm and stained H.E, PAS, PAS- Alcian Blue.

Results

Optical tectum (TO) is a key center for processing sensory information and contains between 11 and 15 distinct morphological cell types, most (over 90%) being piriform neurons. Ventral to it, below the tectal commissure, in the tectal ventricle (V3), is found torus longitudinalis (TL). Surrounding the posterior tip of the tectum, a thin sheet of cells seals the ventricle that extends between the caudal tip of the tectum and the cerebellum (Fig. 1).

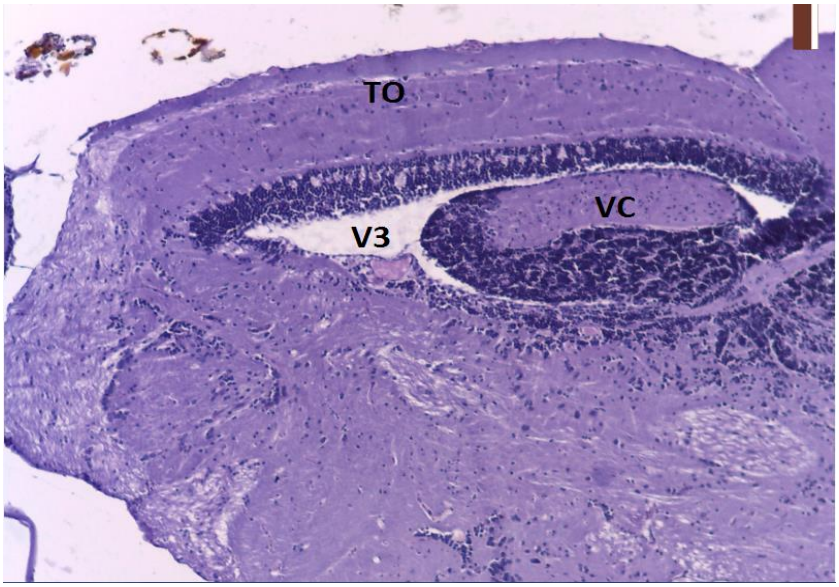

Fig 1. Optical tectum (TO). Ventral, below the tectal commissure, in the tectal ventricle (V3), is found torus longitudinalis (TL) .Col. HE x 40

The areas of the zebrafish tectum as in the case of other teleosts are represented by seven layers that are from the *pia mater* to the ventricle 3, the following: 1-meninges; 2-stratum marginal fibrosum (MS) with non-myelinated axons; 3-layer opticum (SO); 4-stratum fibrosum and superficial griseum (SFGS); 5-stratum griseum centrale (SGC); 6-layer central album (SAC); 7-periventricular stratum (SPV) composed of piriform neurons (Fig. 2).

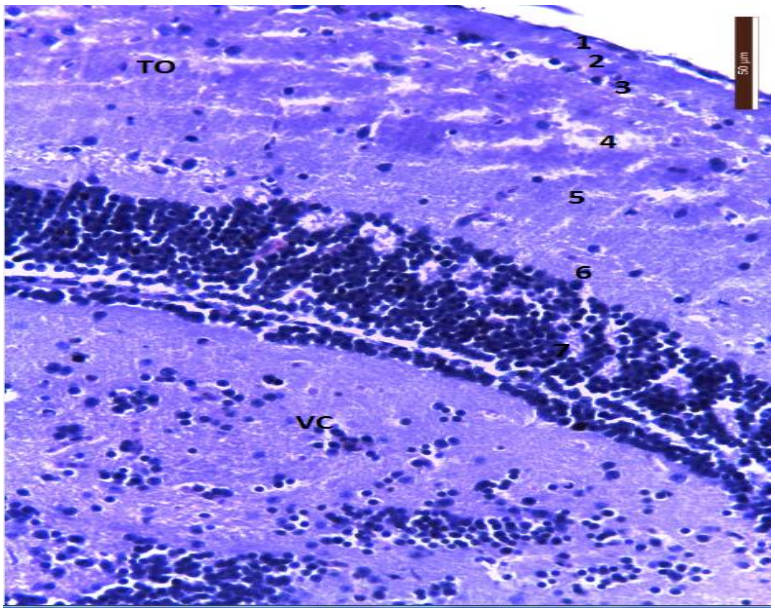

Fig. 2- Optical tectum stratification (TO): 1-meninges; 2-stratum fibrosum marginale; 3-stratum opticum; 4- stratum fibrosum and griseum superficiale; 5-stratum griseum centrale; 6-stratum album centrale; 7-stratum periventriculare, composed from piriform neurons.
Col. Blue Alcian x400

The inputs related to the tectum come from different sensory systems and nuclei. The non-myelinated axons of MS come from the longitudinal torus. SO and SFGS receive most of the retinal terminals. Mechanoreceptive information reaches the SGC and SAC layers of the tectum from the semicircular torus. The telencephalon projects to the GSC (ipsilateral and possibly contralateral). From the diencephalon, numerous pretectal, thalamic and hypothalamic nuclei contribute to the tectum.

Analyzing the caudal division of the three primary divisions of the brain of developing vertebrates, the rhombencephalon, the structure of particular importance is the cerebellum which has a simple laminated architecture consisting of three layers, namely. a molecular layer, a Purkinje cell layer and a granular cell layer (Fig. 3).

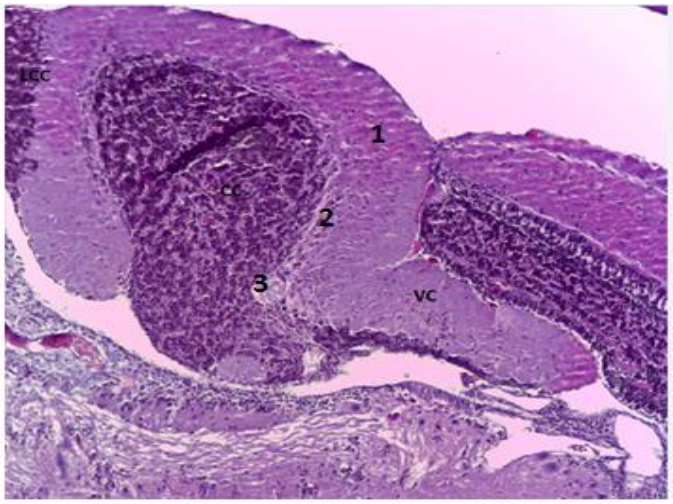

Fig 3- Cerebellum with the 3 structures - corpus cerebelli (CC), cerebelli valve (VC) and lateral vestibular lobe (LCC). The layers of neurons are: 1. molecular layer; 2. Layer of Purkinje neurons; 3. Granular layer. Col. PAS x 40

It has been observed that the granular cell layer consists of excitatory granular cells and inhibitory Golgi neurons and the layer of Purkinje neurons contains Bergmann glial cells and excitatory euridendroid cells. Granular neurons are small and very numerous making this dense. Proliferation of cells are found in all subdivisions of the cerebellum. Although the cerebelli valve extends into the tectal ventricle, it is histologically formed of a granular and molecular layer, to which are added aggregates of large Purkinje cells and euridendroids.

The molecular layer found towards the periphery consists of two types of cells, basket and stellate, which are interneurons. Purkinje cells are among the most complex neurons and are located at the boundary between the two layers (Fig. 4, 5).

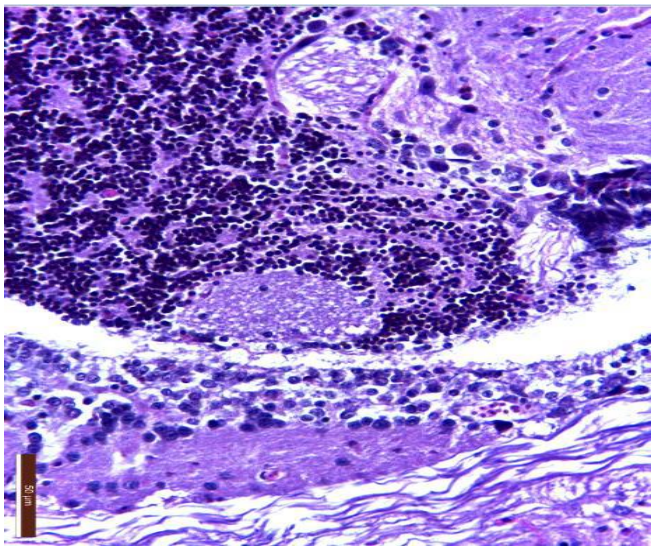

Fig 4- Small, dense granular neurons from the corpus cerebelli.
The central canal is represented by the fourth ventricle - Col. PAS x400

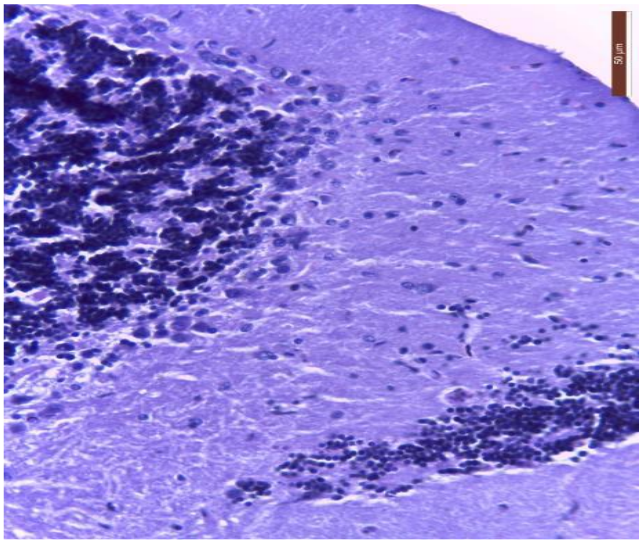

Fig. 5- Cerebellar details- corpus cerebelli. Purkinje neurons and layer of dense, granular neurons.- Col. Blue Alcian x100

In the rostral region, the **myelencephalon** consists mainly of the *medulla oblongata*, as a strain of the brain and the vagus and facial lobes, all of which are connected to the cerebellum at the diencephalon. The central canal as well as the fourth ventricle were found in the middle of the *medulla oblongata*. This region is covered by ependymal cells. The histological details of the *medulla oblongata* highlight oval-shaped neurons and eosinophilic cytoplasm, small glial cells with Nissl corpuscles as well as large circular cells (Fig. 5).

In the spinal cord, the first step toward achieving the diversity observed in adults occurs early in development, with the division of neuronal progenitor cells into distinct domains along the dorsoventral axis in response to signals from local organizing centers. The ventral spinal cord forms four distinct domains, p3, pMN, p1, and p0 (Fig. 7) (Goulding and Lamar, 2000; Jessell, 2000). It is believed that each domain produces neurons that share common features (e.g., axonal trajectories). The next level of complexity appears in each specific area. For example, the p1 domain in mammals produces ipsilateral inhibitory neurons that project ipsilateral with distinct, well-identified functions, such as Ia and Renshaw cells, as well as other ipsilateral inhibitory neurons whose function has not yet been determined (Sapir et al., 2004; Alvarez et al., 2005). Compared to the previous stage of domain formation, the development mechanisms responsible for the complexity resulting from within a domain are less understood.

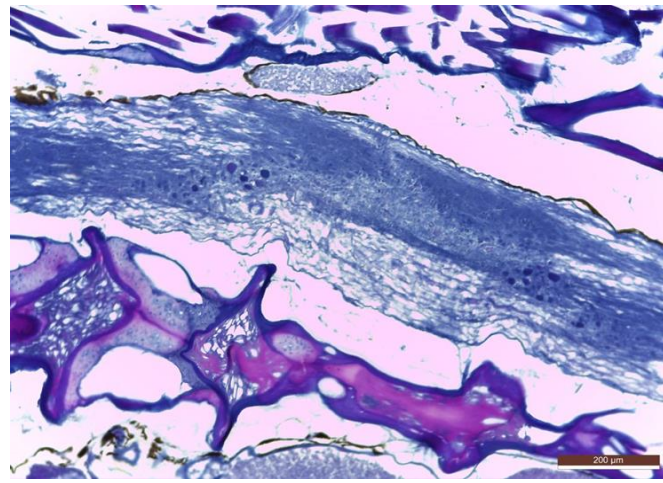

Fig. 6- The medulla oblongata with oval neurons and eosinophilic cytoplasm, small glial cells with Nissl corpuscles as well as large spherical cells. Col PAS-Blue Alcian x100

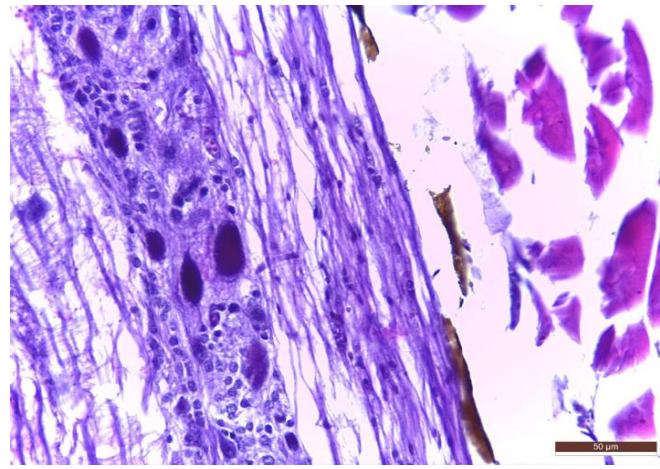

Fig. 7 - The gray matter of the spinal cord is surrounded by the white matter, which contains myelinated nerve fibers. The lumen of the ventricle in the center of the gray matter is lined with ependymal cells. Col. HEA x400

Discussions

Zebrafish is becoming a popular model for studying the structure and function of neural circuits, as it has a variety of advantages over other animal models. Some of these advantages are useful, although not essential, while others allow experiments that are difficult or impossible to perform in other genetic organisms.

Unlike mammals, amphibians and fish do not have a visual cortex (Lázár, 1973; Streidter and Northcutt, 1989). Instead, they have a proportionately larger tectum, which is thought to perform some of the visual processing that the cortex performs in mammals (Nevin et al., 2010; Orger, 2016). The results of this study are in line with the results obtained by Vanegas and col. (1974); Sas and Maler (1986); Meek and Nieuwenhuys (1998) who state that in teleost fish, tectal afferents reach the tectal neuropil, which comprises 7 layers (from outside to inside): the marginal fibrosum layer (MS), which does not receive direct retinal inputs, the opticum layer (SO), fibrosum and superficial griseum layer (SFGS), central stratum griseum (SGC) and central album stratum

and periventricular stratum griseum (SAC / SPV). As stated by Miyamura and Nakayasu (2001), and Wullimann (1996), it has been observed that the cerebellum in teleosts is composed of three major parts: the cerebelli valve, the corpus cerebelli and the vestibulolateral lobe with a three-layer structure, and namely a molecular layer, a layer of Purkinje cells and a layer of granular cells with dense neurons, Purkinje neurons being among the most complex neurons. Goulding and Lamar (2000) that in the ventral spinal cord four distinct domains are formed, p3, pMN, p1 and p0 and the histological details of the oblong medulla consist of oval-shaped neurons and eosinophilic cytoplasm, small glial cells with several nuclei, Nissl bodies as well as large spherical cells.

Conclusions

1. The midbrain is the median vesicle of the brain with 3 main components: the cerebral peduncles, the roof and the optic roof. The tectum has seven layers of fibers with different cell composition.

2. The rhombencephalon controls functions and autonomic balance. Cerebelum, a part of the rhombencephalon has three layers of neurons: molecular, Purkinje and granular cells.

3. Histologically, the medulla oblongata is composed of small oval and glial cells.

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PARTICULARITIES OF OLFACTORY SYSTEM IN ZEBRA FISH (*DANIO RERIO*)

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Abstract
Histological study of olfactory bulbs and peripheric olfactory organs were made on 10 adult zebra fiskes (Danio rerio), both sexes, using paraphin embeding method and HEA and PAS stain. Zebra fish is a very good model for studying the mecanisms for odour detection, olfactory changes of the environement and behavioural effects of them. Olfactory system in zebra fish is formed by a pair of olfactory peripherical organs or rosets, situated in nasal cavity, conected at the olfactory bulbs. Sensorial region of the rosete have an characteristic pseudostratified columnar epithelium consisting mainly from sensitive olfactory neurons. Olfactory bulb is a cerebral structure situated in the most rostral region of the anterior brain, conected to olfactory bulb by a short olfactory nerf. It is the central receptor when the olfactory informations are prepared and sent to telencephalic regions.
Key words: *zebrafish, olfactory system, histology*

Introduction

Danio rerio or zebrafish belongs to the family Cyprinidae, genus Danio, comes from India and has become an important tool in neuroscience research due to its genetic traceability, molecular and physiological preservation, small body size, easy of *in vivo* experimental manipulations and rich behavioral repertoire. Zebrafish models and tests are particularly useful in genetic research, in neurophenotyping, in the screening of CNS drugs, toxicology (Petrovici et all., 2020), as well as in the modeling of complex neurological and psychiatric disorders.

In the last decade, technological evolution has opened up new opportunities to study neural circuits. These include molecular approaches for identifying, labeling, and manipulating certain types of neurons in the brain, studying behavior, advances in extracellular recording techniques to measure the triggering potentials of multiple neurons in animals, and intracellular recording methods. In addition, compared to mammals, the brains of adult zebrafish have a high level and a number of proliferative areas of regeneration. The general organization of the central nervous system of zebrafish is indeed very similar to that of other vertebrates and approaching the nerve segments in an antero-posterior order was observed that it is structured in four areas: forebrain, midbrain or middle brain, rhombencephalon or posterior brain and spinal cord.

The olfactory system, composed from the olfactory organs and the olfactory bulb, allows organisms to interact with their environment by detecting odor signals. Smell mediates behaviors that are essential for survival, such as feeding, mating, social behavior, and hazard assessment. The olfactory bulb (OB) is an associated brain structure located in the most rostral region of the anterior brain, connected to the olfactory organ by a short olfactory nerve; is the central relay of the olfactory system in which olfactory information is processed and transmitted to the telencephalic areas.

Materials and methods

The study was conducted on 10 zebrafish, 5 males and 5 females. The samples taken were the fish entirely, initially placed for 4 hours in 4% formaldehyde. After this prefixation, they were sectioned mid-sagittally and transferred to the Bouin fixator for 48 hours. The pieces were dehydrated, clarified, embedded in paraffin, sectioned at 5µm and stained HEA, PAS, PAS- Alcian Blue.

Results

The olfactory system, composed of the olfactory organs and the olfactory bulb (fig. 1), allows to organisms to interact with their environment by detecting odor signals. The telencephalon of the zebrafish consists of olfactory bulbs, the dorsal telencephalon (TD) or pallium and the ventral telencephalon (TV) or subpallium. The telencephalon consists of solid telencephalic lobes, separated by a T-shaped ventricle. The pallium and subpallium can be divided in two domains (TDM-median dorsal telencephalon, TDP-posterior dorsal telencephalon, TVD-ventral telencephalon dorsal part and TVV-telencephalon ventral part). One of the most distinctive features is that TVV is the only telencephalic region in which cholinergic neurons have been detected. Numerous neuroglia (nvg) were also observed in the structure of the zebrafish telencephalon. The axons in the olfactory bulb form the olfactory tracts in the telencephalon.

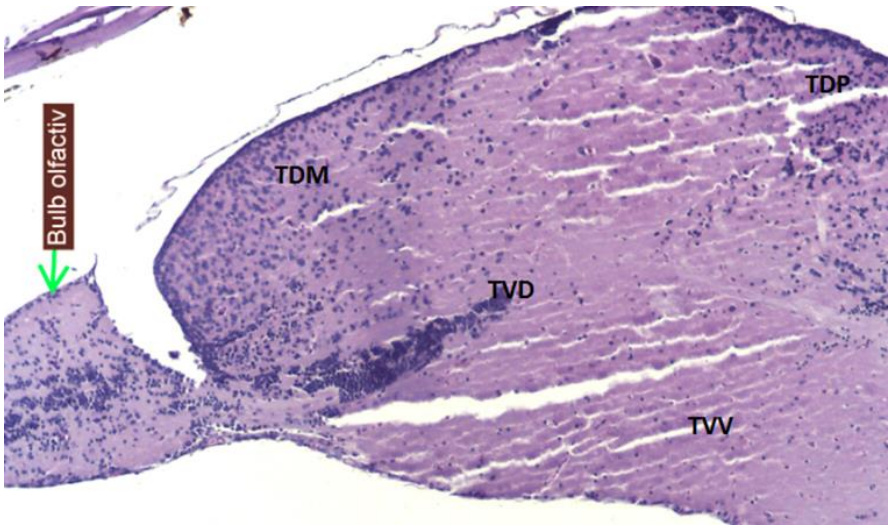

Fig. 1- The telecephalous parenchyma consists of neurons protected by glial cells (astrocytes and oligodendrocytes), as well as microglia and blood vessels. PAS x 40

The general architecture and functional organization of the olfactory system of zebrafish is analogous to that of other vertebrates. It consists of two main structures: a pair of peripheral olfactory organs, or rosettes, located in the nasal cavity connected to the olfactory bulbs (Fig. 2), which is the most frontal region of the rostrum. The olfactory organ of zebrafish contains an epithelium arranged in several lamellae that converge in a non-sensory central area, forming a bilateral, cup-shaped structure known as a rosette.

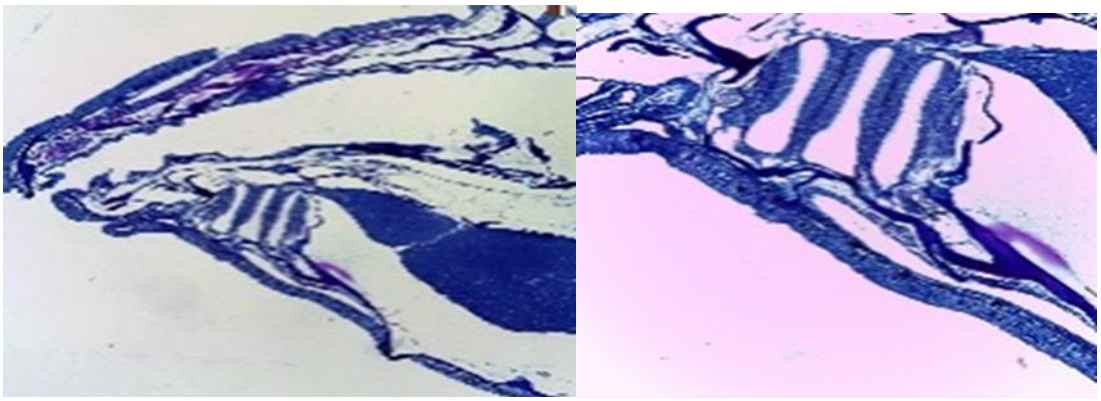

Fig. 2- Structure of the olfactory system of zebrafish: peripheral olfactory organs, or rosettes, located in the nasal cavity connected to the olfactory bulbs. Alcian Blue x100.

The rosette lamellae are composed of a continuous sensory area, located in the central and medial region, as well as a surrounding non-sensory epithelium located dorsally (Fig. 3).

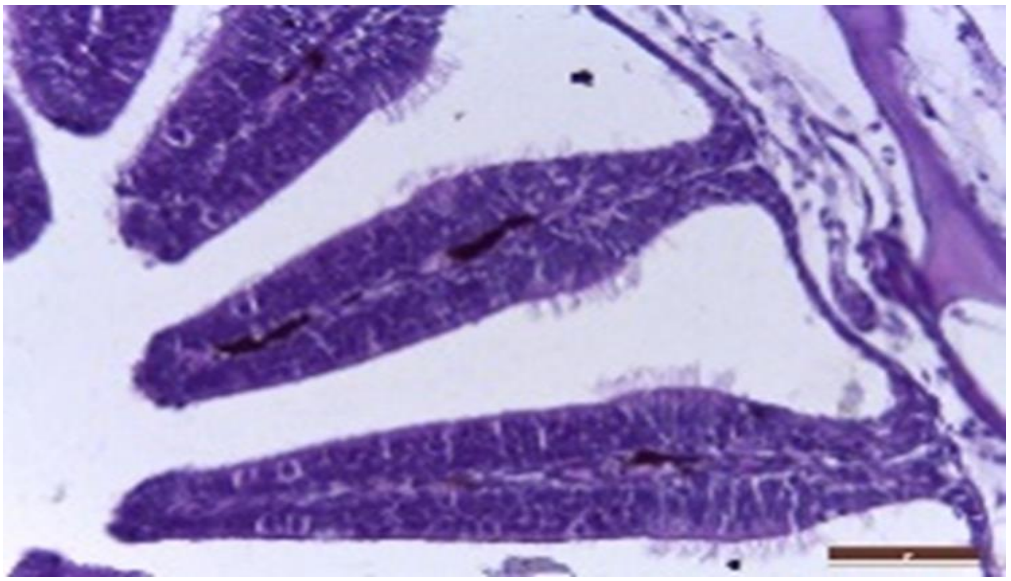

Fig. 3 Peripheral olfactory organs or rosettes, located in the nasal cavity. PAS- Alcian Blue x100

The sensory region of the rosette has a characteristic pseudostratified columnar epithelium consisting mainly of olfactory sensory neurons (OSNs, sensitized by odorants), basal cells and the support cells. There are five types of OSN described in zebrafish (Fig. 4): cills (cl); with microvilli (mv); crypt (cr); Kappe (kp) and pear (pr) neurons described recently. These OSNs have different morphologies, molecular markers and profiles and are differentially located on the entire epithelial layer. Ciliated OSNs are found at the base and have an elongated morphology with a long apical dendrite that continues with cilia. The microvilli OSNs are of intermediate size with apical microvilli that come from a thick dendrite. Crypt neurons are found apically and have a spherical body, appearing as a smaller cell. Kappe OSNs are located on the apical side of the olfactory epithelium (OE) and have a short, globose shape characterized by an apical cap with microvilli.

Pear OSNs, which are also located apically, have a pear-shaped morphology as well as very short apical dendrites (Fig. 5).

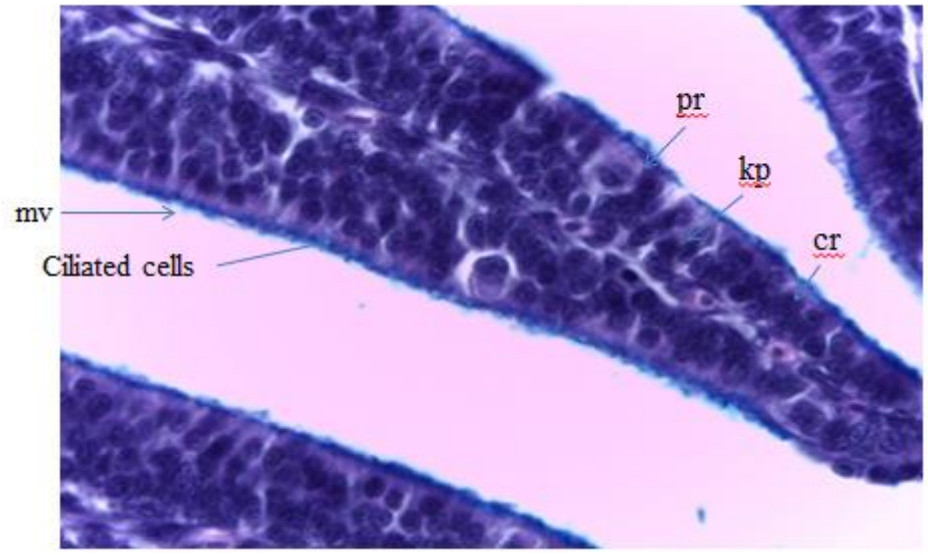

Fig.4- Olfactory epithelium composed of ciliated columnar epithelium with olfactory sensory neurons (OSN): contains microvilli (mv); ciliated cells (cl), cryptic cells (cr); kappe (kp) and piriformis cells (pr). PAS Blue Alcian x1000

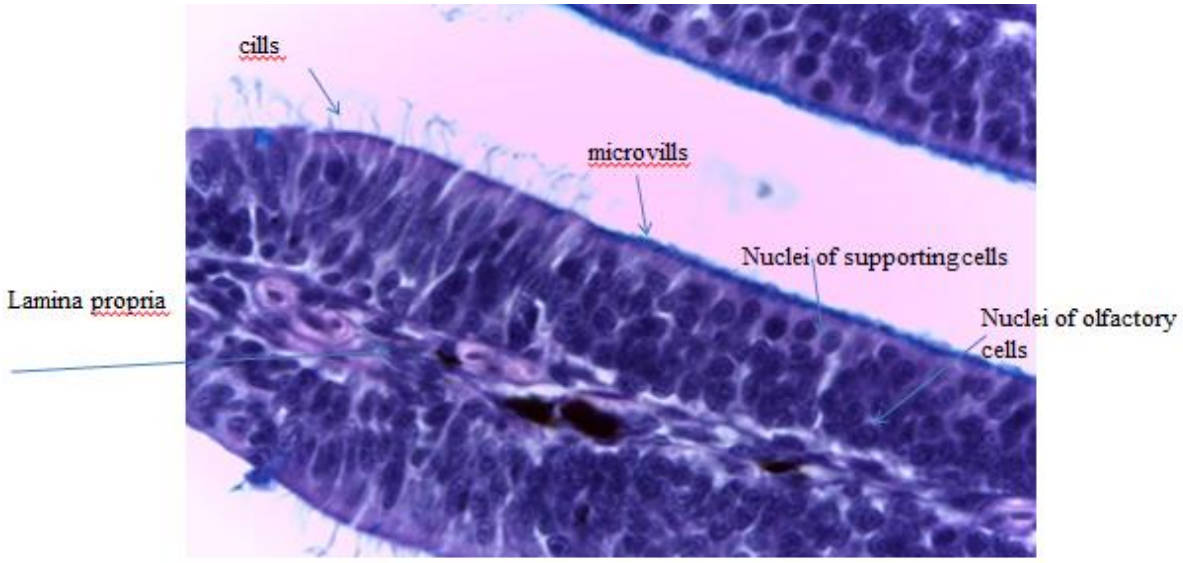

Fig. 5- The layers of olfactory epithelium (OE) with peripheral basal cells with cills and microvills followed by a layer of nuclei with supporting cells and a layer with nuclei with olfactory cells and further in the center is located the lamina propria. HEA x1000

Discussions

OSNs extend the axonal projections to the olfactory bulbs, forming a fasciculate connecting structure known as the olfactory nerve. OSN axons that reach the olfactory bulbs form discrete structures known as glomeruli, where they form synapses with bulbous mitral cells. Glomerular activity patterns are processed by a distributed network of major neurons, mitral cells, and various types of local interneurons, including granular cells, periglomerular cells, and short-axon cells. These neurons extend the axons to the anterior brain in bundles known as olfactory tracts, where they transmit signals to the respective telencephalic olfactory processing areas to the posterior area of the dorsal telencephalon; the ventral nucleus of the central telencephalon; posterior tubercle and right habenula. Olfactory information is processed and decoded in these telencephalon centers to cause odor-mediated behaviors. In addition to having a well-characterized morphology and neural circuits, the olfactory system of zebrafish has remarkable mechanisms of regeneration, repair and reorganization in the basal states in response to injury. Both the olfactory organ and the olfactory bulb show continuous neurogenesis and neuronal fluctuation throughout the body's life. These characteristics make the olfactory system of zebrafish an ideal model to study the mechanisms of olfactory processing, olfactory dysfunction and regeneration after deterioration.

While ciliated and microvilli cells are also present in higher vertebrates, cryptic cells have been found only in fish. In zebrafish, homologous circuits consist of usually from far fewer neurons than in mice. The olfactory bulb, for example, contains approximately 500 neurons in zebrafish larvae and 20,000 to 30,000 neurons in adults (Mack-Bucher et al., 2007; Wiechert et al., 2010), compared to ~ 10^6 - 10^7 neurons in grown up mices. Therefore, zebrafish allow the sampling of neural activity on a large fraction of neurons in many areas of the brain. Limited sampling is sufficient when the responses are dense and when a calculation can be explained by simple statistical properties of neural activity patterns. For example, the responses of individual neurons in the sensory brain areas are often reduced depending on the average input through an operation called “normalization”. Dense sampling may be required, for example, to define the state of a network, especially when these states are not triggered by an external event, but occur spontaneously. In general, dense sampling becomes important when neuronal activity itself is reduced and when information processing depends on specific subgroups of neurons. Dense measurements and detailed neuron-by-neuron analyzes of activity patterns may therefore be necessary for a rigorous perspective on important neural calculations. Circuits whose function depends on poor activity and the specific structure of activity patterns are likely to be common in vertebrates, for example, in the cortex and cerebellum.

Zebrafish provide a favorable model for studying both the mechanisms underlying the detection of odor and olfactory changes, as well as their behavioral effects.

Conclusions

1. The olfactory system is composed of the olfactory organs and the olfactory bulb and allows organisms to interact with the environment.

2. The olfactory organ consists primarily of a sensory epithelium represented by olfactory sensory neurons (OSNs) that respond to odor or odorant molecules.

3. OSNs are bipolar sensory neurons that extend from the basal lamina to the apical region of the epithelium, where they detect odors in water and are of five types of cells: with microvilli, ciliated, cryptic kappe (kp); and piriform.

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HUMANIZED MOUSE MODELS AND HUMAN VIRUSES

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Abstract
Well-developed mouse models are important for understanding the pathogenesis and progression of immunological response to viral infections in humans. Moreover, to test vaccines, anti-viral drugs and therapeutic agents, mouse models are fundamental for preclinical investigations. Human viruses, however, seldom infect mice due to differences in the cellular receptors used by the viruses for entry, as well as in the innate immune responses in mice and humans. In other words, a species barrier exists when using mouse models for investigating human viral infections. Developing transgenic (Tg) mice models expressing the human genes coding for viral entry receptors and knock-out (KO) mice models devoid of components involved in the innate immune response have, to some extent, overcome this barrier. Humanized mouse models are a third approach, developed by engrafting functional human cells and tissues into immunodeficient mice. With an increase in the advancement of modern techniques used for genetic manipulation, humanized mice have become an important asset. They are becoming indispensable for analyzing human viral diseases since they nearly recapitulate the human disease. These mouse models also serve to test the efficacy of vaccines and antiviral agents. The development of humanized mouse models offers a preclinical in vivo platform for further characterization of human viral infections and human immune responses triggered by these virus particles. This review highlights recent progress in utilizing humanized mice to decipher human specific immune responses against viral tropism.
Keywords: *infectious diseases, human viruses, mouse models, transgenic mice, humanized mice*

1. Introduction

The use of small animal models such as mice and rats has contributed greatly to the understanding of disease pathogenesis and development of therapeutic approaches. Basically, these animals act as surrogates in representing human biology due to the limitations and ethical restrictions of obtaining tissue samples directly from human donors for research purpose. Moreover, these mammalian model systems are often easier to maintain and handle due to their nature of being small, have a high reproductive turnover, and share similar genomic and physiological characteristics with that of a human. Despite utilizing these amazing properties for basic biology, a fine line still separates mice studies from humans as they lack an integral component required in the human microenvironment herein, the immune system. For instance, it is known that the innate immune responses differ between man and mouse whereby mice lack a functional Toll-like-receptor 10 (TLR10) whereas TLR11, TLR12, and TLR13 which are expressed in mice are actually absent in the human genome (1, 2). Moreover, immune responses in wild-type mice infected with murine-adapted viruses are completely different to human immune responses triggered by human-specific pathogens due to inter-species diversity although they are used in studying the same virology. "Humanized" mice with functional human cell/tissue engraftment have garnered some interest lately and are gradually being recognized as an in vivo prerequisite in bridging the gap from bench-to-cage-to-bedside. However, it was not until the early 2000s when immunodeficient mice bearing mutations at the interleukin-2 receptor common gamma chain (*IL-2ry^{null}*) were used for efficient human cells and tissues engraftment (3,4). This proved to be a major breakthrough as the absence of *IL-2rγ* led to severe impairments in multiple cytokine complexes involving IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 signalling and ultimately profound T cell defect. When these *IL-2ry^{null}* mice were backcrossed with either the protein kinase DNA activated catalytic polypeptide mutation (*Prkdc^{scid}/scid*) or with recombination activating gene

(*Rag*) 1 or 2 (*Rag1*^{null} or *Rag2*^{null}) mutations, murine adaptive (T and B cells) and innate natural killer (NK) cells immunity were completely compromised including defects in mouse macrophages and some dendritic cell subsets (5).

2. Models of Human Diseases Established on Humanized Mice

The introduction of humanized mice provides immeasurable opportunities to advance medical research. These increazingly important pre-clinical models are not only easy to handle due to their small sizes, but they also have short reproductive cycles, an exceptional ability to produce a large number of young and are relatively affordable to maintain in animal facilities as they do not require highly specialized infrastructures that are used by NHPs. In addition, humanized mice allow human-specific pathogens to infect and replicate within them and are able to develop functional human-specific immune responses to an array of diseases.

2.1. Flaviviruses

Members of the virus family Flaviviridae other then DENV include yellow fever virus (YFV), west Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and Zika virus. Although non-human primates (NHPs) like Rhesus and/or cynomolgus macaques remain the best model for hosting DENV, YFV and Zika viruses naturally for vaccine development, the lack of resources in assessing immune responses such as detection of antigen-specific T cell responses after vaccination proved to be a limitation in NHP models. Therefore, humanized mice were utilized not only for antibody production but also immunophenotyping of specific immune subsets. In fact, humanized monoclonal antibodies have been reported for viral neutralization but not clearance against YFV and JEV in vivo. On the other hand, both WNV and Zika virus tackled the advantages of the human immune system in humanized BLT mice for further viral characterization and antiviral therapeutics. Notably, these BLT mice displayed persistence Zika viremia of up to 7 months post-infection which was attenuated with neutralizing antibody.

2.2. Hantavirus

Hantavirus, a negative-sense RNA virus in the Hantaviridae family, is another type of infectious disease that can cause haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) which give rise to increased vascular permeability and loss of platelets. Rodents generally serve as natural hosts for hantaviruses but it can be detrimental in humans. Hantavirus replication was observed in cell culture but does not have cytopathic consequences suggesting that the human immune system is required for induction of HFRS or HCPS (6, 7).

2.3. Influenza

The flu virus that makes up part genera of the family *Orthomyxoviridae* is one of the most common causes of human respiratory infections (8). Although vaccines and antiviral drugs are available to prevent or treat influenza respectively, there is no guarantee that one could escape infection entirely due to the constant evolution of different viral strains. Some of the mild symptoms include high fever, runny nose, coughing, sneezing, sore throats, etc. but complications may lead to more severe outcomes like gastroenteritis, pneumonia and even deaths. The use of small animal models like humanized mice would allow further understanding of influenza viral life cycle as well as viral replication which ultimately led to some of these symptoms. Indeed, humanized mice represent the best model for studying the flu virus but its poor development in the myeloid compartment remains a major drawback for triggering an immune response at mucosal surfaces such as the lungs. Many reports have demonstrated that human cytokines, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are essential for pulmonary homeostasis, myeloid cell development and host defense against pathogens. Thus,

immunodeficient mice transplanted with $CD34^+$HSCs were generated with human cytokines knock-in of IL-3 and GM-CSF to compete against mouse cytokines. Substantial improvements in the development of alveolar macrophages triggered effective innate responses when challenged with influenza virus. These humanized mice consistently express high amounts of GM-CSF, tumor necrosis factor alpha (TNF-α), and IL-6 mRNA in the lungs after infection. In addition, M-CSF treatment following influenza infection in NSG Hu-SRC-SCID mice similarly displayed decreased viral transcripts which was associated with overproduction of proinflammatory cytokines, TNF-α and IL-6.

3. Hepatotropic Pathogens in Human Liver Chimeric Mouse Models

The immune system is essential for human immune surveillance for viral replication and providing specific immune biomarkers for viral clearance of all human pathogens. Listed above are some of the human-associated viruses known to have been investigated in humanized mice. However, the study of hepatotropic pathogens remained elusive due to the lack of human hepatocytes in the mouse liver. As mentioned earlier in this review, the establishment of two commonly used human liver chimeric mouse models, Alb-uPA transgenic mice and *FRG* KO mice, fully support the viral life cycle which led to further characterization of infectious pathogens (9,10).

3.1. Hepatitis C Virus (HCV)

Hepatits C virus (HCV) is a single-stranded enveloped flavivirus that binds to cell surface in order to release virus particles into cells by receptor-mediated endocytosis. The restriction of HCV tropism to humans proved to be a major obstacle in understanding viral–host interactions, HCV-specific immune responses, disease progression, and identification of novel drug candidates. The Alb-uPA and *FRG* KO chimeric mice mentioned earlier were first models to exhibit localization of HCV viral proteins in human hepatocytes nodules. HCV from mouse serum was also serially passaged and infected into 3 generations of mice confirming both synthesis and release of infectious viral particles. Transgenic mice expressing these key human-specific factors facilitated viral uptake and replication which was suppressed by both innate and adaptive immunity in vivo. To better understand the role of the human immune system in targeting HCV, humanized mice generated by co-transplantation of $CD34^+$HSCs and hepatic progenitors into hAlb-FKBP-Caspase 8 (*AFC8*)$^+$ transgenic mice supported HCV-induced immune responses and liver diseases. HCV-infected mice displayed elevated levels of human $CD45^+$ leukocytes including $CD68^+$ macrophages and $CD3^+$ T cells infiltration in the liver. These mice also developed severe liver fibrosis but not in immunocompromised Alb-uPA and *FRG* KO chimeric mice after HCV infection indicating the importance of having a fully functional immune system to trigger liver damage.

3.2. Hepatitis B Virus (HBV)

Like HCV infection, chronic HBV (a member of the *Hepadnaviridae* family; genus *Orthohepadnavirus*) can also cause liver inflammation/fibrosis which gives rise to liver cirrhosis and/or ultimately HCC in patients. Although HBV vaccines are available, it is not a solution for established infections. The majority of antiviral therapies could suppress viral replication but not eradicate HBV entirely due to the stability of the covalently closed circular DNA (cccDNA). Indeed, human liver chimeric mice remain the gold standard for supporting hepatotropic infections; however, the highly immunocompromised status of these engrafted mice precludes liver pathogenesis mediated by human immune surveillance. Hence, dual humanization of liver and the immune system was established in immunodeficient mice to study immune responses and liver disease progression in the context of HBV infection. his dual humanized mouse model system allowed persistent HBV infection over several months followed by liver inflammation and fibrosis facilitated by M2-like macrophage infiltration suggesting a critical role for macrophage

polarization in HBV-induced impairment and liver pathology. Similarly, another group demonstrated dual humanization of liver and immune system by syngeneic engraftment of human hepatoblasts and HSCs in *Fah* KO NOD *Rag1*^{null}*/IL-2ry*^{null} (FNRG) mice which also portrayed rapid and sustained viremia upon HBV infection. Similarly, another group demonstrated dual humanization of liver and immune system by syngeneic engraftment of human hepatoblasts and HSCs in *Fah* KO NOD *Rag1*^{null}*/IL-2ry*^{null} (FNRG) mice which also portrayed rapid and sustained viremia upon HBV infection. Due to the limited access to fetal tissues, an alternative method of dual humanization of the liver as well as the immune system was established via transplantation of mature hepatocytes and HSCs from different donors. Here, HBV-infected humanized mice exhibited partial immune control over viral life cycle as evidenced by presence of antigen-specific IgGs and liver-infiltrating Kupffer cells, NK cells (CD69^{+}) and PD-1^{+} effector memory T cells which was in line with immunopathology observed in patients with chronic HBV. Plasma from these infected mice also displayed elevated levels of inflammatory and immune-suppressive cytokines, C-X-C motif chemokine ligand 10 (CXCL10) and IL-10 which correlated with the intrahepatic CD4^{+} T cells subset (11, 12, 13).

3.3. Hepatitis E Virus (HEV)

Hepatitis E virus (HEV) is a single-stranded, non-enveloped RNA icosahedral virus comprising a positive-sense, single stranded RNA genome which transmits viruses via the faecal–oral route. Although acute HEV infection is quite common, patients often undergo full recovery following antiviral treatments. Animal studies of HEV infection are limited but human liver chimeric mice remain the best model for further viral characterization. Since only the mouse liver is humanized, administration of HEV had to be performed intravenously or via the mouse spleen rather than orally due to species-specific intestinal host restrictions. A full viral life cycle was established in HEV-infected mice as evidenced by detection of viral RNA in faeces, bile and liver. Mice with humanized liver harbored the virus over several months without hepatotoxicity but displayed increased expression of innate genes like CXCL9, CXCL10, HLA class 1, and ISGs which may be mediated by the immune response. However, just like HDV research, human-specific immune responses targeting HEV infection are yet to be explored in vivo. Nevertheless, antiviral therapeutics using Ribavirin have shown efficient reduction of virus titer in both plasma and faeces of HEV-infected human liver chimeric mice (14).

4. Conclusions

The evolution of humanized mouse models has contributed to better characterization of human infectious diseases and immune responses. Only in recent times have humanized mice been utilized more frequently as a preclinical platform for in vivo validation, but key improvements in specific areas are required. Firstly, the study of murine-adapted viruses in wild-type mice may not necessarily recapitulate an infectious phenotype observed in clinical settings, suggesting that the source of species-specific virus inoculum is critical for fully understanding viral life cycles and replication/production capabilities in humanized mice. Therefore, the establishment of a fully functional human immune system and/or liver system in vivo is required to accommodate such human-specific infectious pathogens. Reconstitution of human immune cells permits the study of blood-borne viruses while repopulation of human hepatocytes in human liver chimeric mice is essential for the study of liver-associated infectious diseases like viral hepatitis. Most importantly, dual humanization of liver and immune system was able to recapitulate liver pathogenesis and trigger immune responses similarly observed in patients. Secondly, although human immune profiling can be investigated in humanized mice when challenged with infectious pathogens, the less optimal cell reconstitution and responses of the human innate immune components in

humanized mice remain a major limiting factor. New strains of immunocompromised mice have been generated by further replacement of murine immune counterpart components with human histocompatibility markers and other human specific molecules e.g. human cytokines via transgenic knock-in approaches to improve engraftment and maturation of human immune subsets. Other efforts to improve human immune reconstitution in mice have been enforced through various methods like expansion and differentiation of $CD34^+$ HSCs in vitro prior to mice injection, gene editing technology, packaging and delivery of human cytokines.

Hence, technological advancements in humanized mouse models have allowed more robust in vivo characterization to further elucidate viral–host interactions and identify novel immunotherapies and/or vaccine strategies.

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MOUSE MODELS AND SARS-COV-2

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Abstract
Since December 2019, a novel coronavirus SARS-CoV-2 has emerged and rapidly spread throughout the world, resulting in a global public health emergency. COVID-19 is causing a major once-in-a century global pandemic. The emergences of coronaviruses have caused a serious global public health problem because their infection in humans caused the severe acute respiratory disease and deaths. Much more serious than SARS-CoV in 2002, the current SARS-CoV-2 infection has been spreading to more than 213 countries, areas or territories and causing more than 31 million cases and 962,518 deaths (update september 2020). Intensive research efforts have focused on increasing our understanding of viral biology of SARS-CoV-2, improving antiviral therapy and vaccination strategies. The lack of vaccine and antivirals has brought an urgent need for an animal model. The animal models are important for both the fundamental research and drug discovery of coronavirus.
Keywords: *coronavirus, respiratory syndrome, animal model, vaccine, drug*

1. Introduction

Animal models are critical for us to understand the viral infection and pathogenesis. Moreover, animal models are essential for development and preclinical evaluation of a vaccine or an antiviral agent. An ideal animal model is the one that mimics viral infection and diseases in humans in multiple aspects including morbidity, viral load, typical clinical symptoms, host immune responses and mortality. Therefore, the urgent need of preventing and controlling coronavirus infection necessitates the search for an optimal SARS- CoV-2 animal model. Based on the published studies, animal models of SARS-CoV and MERS-CoV include civet cats, camelidaes, monkeys, mice, hamsters, ferrets, rabbits and other potential hosts. Humanized animal models available to support coronavirus infection and pathogenesis might provide new options to overcome the limitations of the traditional coronavirus animal models. Additionally, animal models for pseudovirus are also prospected to avoid the concern of biosafety.

2. Emerging coronaviruses infections

Clinical symptoms of SARS-CoV- and MERS-CoV- infected patients at early time include fever, chills, coughing, malaise, myalgia, headache, diarrhea, vomiting and nausea. Furthermore, immunohistochemistry (IHC) detection demonstrated the presence of viral antigens in lung tissues. The COVID-19 patients present similar symptoms to those of SARS- CoV- or MERS-CoV-infected patients, while some patients may show no typical clinical symptom in the early stage of infection. The typical pathological features of severe cases include prolonged inflammation with destruction and desquamation of alveolar pneumocytes, hyaline-membrane formation, interstitial inflammatory infiltration and interalveolar hemorrhage. Multinucleated giant cells were also observed in the tissues of COVID-19 patients. Over 70% of COVID-19 patients were diagnosed as pneumonia by chest computed tomography (CT) to be admitted to hospital. CT images showed the typical features of ground-glass opacity and bilateral patchy shadowing in lungs. Recent clinical and experimental studies have demonstrated SARS-COV-2 caused the nosocomial infection and fecal-oral transmission, its infection results in immune abnormality and multiple organ failures of the COVID-19 patients. Moreover, a long incubation period (>29 days) was observed in some

COVID-19 cases, suggesting a risk of occult and chronic infection. Although SARS-COV-2 displays a lower rate of severe cases and case fatality than SARS-CoV and MERS-CoV, its high infectivity, widely spreading and huge infection population have become a catastrophic medical burden and critical social problem (1, 2, 3).

3. Natural Infectious animal models

Viral entry and intracellular replication are two essential steps for a virus to establish a successful infection. The specific receptor proteins anchored on cell mem- brane mediate viral entry and intranuclear transcriptional factors regulate viral replication. Therefore, host-range of virus depends on these two steps. Non-human primates (NHPs), ferrets and hamsters have been demonstrated to support SARS-CoV infection and pathogenesis, but only the NHPs are fully permissive for MERS-CoV infection. The civet cats were demonstrated permissive for SARS-CoV infection by serological antibody detection. The camelidaes have been demonstrated permissive for MERS-CoV infection because the specific antibodies against MERS-CoV S protein were detected at a high rate, and respiratory infections after the administration of MERS-CoV. Immunocompetent young inbred mice support transient SARS-CoV infection without clinical signs of disease, but were not permissive to MERS-CoV due to the lack of functional receptor. After a careful review of their illness, virological, histological and immunological characteristics post infection, NHPs, ferrets and hamsters have been presumed to be potential natural hosts of SARS-CoV- 2. Meanwhile, other experimental animal models such as tree shrew, woodchuck, pangolin, rat, guinea pig and cotton mouse, as well as domesticated animals such as cats and dogs might be potential hosts to support SARS-CoV-2 infection (1, 2, 3, 4, 5, 6, 7, 8).

4. Mouse models

Mouse model has been widely used for many different viral investigations. It has been considered as the best small animal model for hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Zika virus, and among others. Due to its low cost, small size, easy operation and high reproducibility, mouse model is suitable for large scale studies of viruses not only for the pathogenesis but also for antivirals. In contrast of the NHPs, mice are more convenient to be handled in a higher level of biosafety laboratory due to its relatively small size and less operation difficulties. Importantly, mouse can be easily manipulated at the genetic level for precision research. For instance, a lot of genetically mutated mice are available for studies in anti-viral immunity, viral pathogenesis and viral infection and transmission restriction. Furthermore, current murine immunological reagents are available to study viral pathogenesis and host immune responses. Several strains of mice have been tried to be intranasally infected with SARS-CoV (Urbani strain), it was found that the virus poorly replicated in several young and adult inbred strains of mice (BALB/c, C57BL6 and 129S) to only a low viral yield. Clinical illness was not observed, and the virus was cleared within nine days. Meanwhile, mice are not naturally susceptible to MERS-CoV infection. It was later revealed that the mouse DPP4 receptor differs from human counterpart in crucial domains that is critical to bind the S protein. To overcome the species-specificity barrier for SARS-CoV or MERS-CoV, human receptor protein (hACE2 or hDPP4) was expressed in mice by knocking in or transfecting a human receptor gene in mouse. The studies of hACE2-transgenic mice were performed at almost the same time in 2007 independently in three groups. They reported that hACE2- transgenic mice supported SARS-CoV infection and pathogenesis. McCray et al. demonstrated that systemic expression of hACE2 under the control of an epithelial cell-specific promoter K18 resulted in lethal SARS-CoV infection within five days. Tseng et al. developed two lineages of transgenic mice expressing hACE2 under the CAG promoter. After SARS-CoV infection, the two transgene-positive mice, AC70 and AC63 showed

high viral load, clinical illness and tissue pathology. The lethal lineage of mice (AC70) displayed higher hACE2 expressions in several organs, wider spectrum of clinical illness, including wasting symptom, severe pneumonia and death, rather than the non-lethal lineage mice (AC63) with lower hACE2 expressions. Yang et al. generated another non-lethal lineage mouse that expresses hACE2 under mouse ACE2 promoter. Another study further revealed the differential virological and immunological outcomes among different hACE2 transgenic lineages.

To rapidly generate a MERS-CoV infecting mouse model, Zhao et al. transduced common adult BALB/c and C57BL6 mice with an adenoviral vector expressing hDPP4 (Ad5-hDPP4). After MERS-CoV infection, these mice showed interstitial pneumonia, expressed viral antigen in the lungs and lost weight, but deaths were not observed. Later, hDPP4-transgenic mice were generated to mimic severe MERS-CoV infection and lethal pathogenesis. Agrawal et al. developed a transgenic mouse that expresses hDPP4 under the control of the CAG promoter. This hDPP4-transgenic mouse was fully permissive for MERS-CoV infection to young adult BALB/c and C57BL6 mice might provide an avenue for rapid and robust generation of SARS- CoV-2 infectious mouse model.

Several studies have attempted to use the immunecompromised mice to determine the role of immune effectors in the coronavirus infection. First, the following knock-out mice have been applied for the infection of SARS-CoV: beige lacking functional NK cells, $CD1^{-/-}$ lacking NK-T cells, $Rag^{-/-}$ lacking T and B cells. However, all the immune-compromised mice failed to allow the infection of SARS-CoV. Viral kinetics were not significantly different among the 57BL6 (wild type), beige, $CD1^{-/-}$ and $Rag1^{-/-}$ mice. Histopathological detection showed similar self-limiting bronchiolitis and mild pneumonia among these mice. Interestingly, a prolonged viral replication and illness were observed in $STAT1^{-/-}$ mice with 129S back- ground. In this model, viral replication is detectable until day 22 post infection indicating that a STAT1 mediated type I interferon response is required to control SARS-CoV infection. The $STAT1^{-/-}$ mice were also challenged with MERS-CoV (EMC- 2012 strain). Because the lack of receptor hDPP4, clinical illness were not observed and viral replication was undetectable. However, the hDPP4-transfected mice additional deficiency of interferon-α receptor, MyD88 and MVAS still cannot prolong the viral replication or cause severe cause throughout a MERS-CoV infection course. Generally, mice with targeted immune deficiency are useful tools to investigate interaction between host immunity and coronavirus. Besides the relatively low viral load and mild illness, the immunodeficient mice are of limited value in the studies of vaccine and immunotherapy (9, 10, 11, 12, 13, 14, 15).

5. Humanized animal models for studying coronavirus infection.

Prevention of and recovery from the coronavirus infection are usually associated with adaptive and innate immunity. The outcomes of the coronavirus infection depend on humoral immune responses such as the subtype and titre of antibody. Although NHPs, mice and other experimental animals can effectively mimic coronavirus infection, the genetic diversity might disturb the interpretation of the possibly different results between human and non-human species. To directly study coronavirus infection in human organ or tissue, humanized animal models are the method of choice, which can also be used for evaluation of coronavirus vaccines and host target agents. In the past decades, mice with one or several human tissues or cells engraftment have been widely used to investigate the pathogenesis of HIV, HBV, HCV, CMV, varicella-zoster virus (VZV) and other important pathogens. Lung is the main target organ of coronavirus infection. In 2012, Maidji et al. grafted human fetal lung tissues under the kidney capsule of immunodeficient SCID mice. The lung tissues rapidly grew and developed mature structures resembling the normal human lung and were demonstrated to support CMV infection and pathogenesis. In 2017, Wang et

al. established a human lung-xenografted mouse model for VZV infection. After VZV infection, viral replication, lung pathogenies and pro-inflammatory cytokine responses were detected in the human lung xenografts. More recently, Wahl et al. reported a humanized mouse with mono human lung engraftment (LoM) or incorporated with human bone marrow, liver and thymus (BLT-L). The LoM or BLT-L mice supports infection and replication of human pathogens such as MERS-CoV, RSV, CMV and Zika virus. Antigen-specific humoral and T-cell responses were observed in BLT-L mice, suggesting that human lung and immune cell dual chimeric mice might be an ideal humanized animal model for the pathogenesis and immune study of SARS-CoV-2. Due to the abundant expression of hACE2 in multiple organs of human, multiple organs and tissues including liver, heart, intestine, kidney, bladder and immune cells are susceptible to the infection of SARS-CoV-2, suggesting a reason that causes multi-organ failures. Therefore, mice with engraftment of human somatic, progenitor and stem cells, as well as varied human tissues and organoids might be useful to investigate the tropism of SARS-CoV-2.

Comparing to the traditional animal models, the humanized mice provide new options for investigators to directly study the viral infection in human tissues, which is adequate to delineate the tissue tropism and the host-virus interactions. The development of humanized mice is important to improve our fundamental understanding for mechanisms of coronavirus infection and immunopathophysiology. In the future, humanized mice might become a unique tool to obtain the insights into our strategies for developing coronavirus vaccine, early intervention and antiviral therapy.

6. Conclusions

The coronaviruses are one of the most important pathogens causing robust respiratory infection, severe cases and deaths in humans. A rapid animal test of SARS-CoV-2 is important to assess the efficiencies of the vaccines, antivirals and the sensitivity of the diagnostic tests. We also debated that a rapid generation of MA viral strains or mice carrying human receptor is a good option for urgent and effective animal studies. In addition, development of humanized animal model might provide a direct infection of coronavirus to human tissue. Taken together, animal models are the fundamental tolls to investigate the viral pathogenesis, to develop vaccines and antiviral drugs.

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PERFORMING A RENAL BIOPSY IN DOGS

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Abstract
Performing a renal biopsy has its reasons in establishing a specific diagnosis, evaluating the severity of the renal lesions and establishing an etiological treatment. Kidney diseases have a high incidence rate in dogs and cats. In order to make a differential diagnosis of a chronic or acute renal insufficiency or of a glomerular lesion , the anamnesis, clinical examination and lab results are not always enough. Renal biopsy is always necessary to establish the degree of severity, a definitive diagnostic and a treatment plan. The succes of the treatment is related to knowing the type and extension of the lesion. The literature indicates that the risks and complications that follow a renal biopsy are extremely low. Still, how we choose the patient and the proper technique may considerably decrease the risk and increase the succes rate of the biopsy. In the final stages of kidney disease, assesed through clinical and laboratory exams, renal biopsy is useless. In the less advanced stages the diagnosis offered by a histopathological examination of the biopsy fragment may proove to be an important factor in achieving a succesfull treatment of the glomerular illness in both dogs and cats. The studies that have approached this subject show that kidney biopsy performed in patients that suffer from a chronic renal insufficiency are not recommended, due to the fact that the risks are higher.
Key words: *renal biopsy, recommendations, risk*

General recommendations before considering a renal biopsy

Generally, it is recommended that before we perform a renal biopsy we consider the history of the patient, the results of the physical exam, its blood presure, biochemical profile, blood profile, urine analysis and blood clotting parameters.

Situations when kidney biopsy is not recommended include coagulopathies, severe anemia, hydronephrosis, arterial hypertension, large or multiple renal cysts, perirenal abscesses, severe pyelonephritis and "end-stage" kidney. In patients that only have one kidney, renal biopsy may sometimes be an option, as long as there isn't any other reason against it. Also, any urinary tract infections are to be treated before this procedure. Performing a biopsy on a kidney suffering from severe hydronephrosis is not recommended because the deep punctioning down to the renal bassinet, already distended by urine under pressure may cause its release, but also because there is a high risk of encountering blood vessels from the medulary area.

Identifying patients with a tendency towards bleeding (coagulopathies) is imperative when selecting a candidate for a biopsy.

Ultrasound guided biopsies are not recommended in dogs with severe thrombocytopenia (≤80000/µl), extended prothrombine time (OSPT> 1,5 x normal), increased leves of nitrogen in the blood stream (serum creatinin >5 mg/dl), uncontroled arterial hypertension or those to whoom were administered non-steroidal anti inflammatories for the past 5 days. Still, when biopsy is absolutelly necessary, the animal must be monitorized in order to prevent possible perirenal hemorrhages and a blood transfusion protocol may also be prepared in stand by.

From a technical point of view, biopsies may be performed either transcutaneously or surgically. The first ones may be done using a laparoscope, an ultrasound or blind.

No matter the method, the biopsy should concern only the renal cortex, as the renal medular area is constituted mainly of tissues abundant in blood vessels, thus risking a large hemorrhage, infarcts or fibrosis.

For the biopsy it is recommended to approach the cortical area through either the apical or the caudal pole. The right kidney is prefered due to the fact that it is more stable (less floatant), being more attached to the caudate lobe of the liver.

Sedation and anaesthisia

Performing a biopsy presuposes a prior restraining of the animal. In order to achieve a good immobilization of the animal anaesthesia or sedation is recommended. In animals with a good health status, we recommend general anesthesia, whilst in those with a precareous health status sedation is more suited.

Choosing the percutaneous biopsy needles is done according to the patient and biopsy gun (fig. 1). The size may vary (14G, 16G, 18G, 20G with lenghts of 6, 9, 15 cm) from case to case (fig. 2).

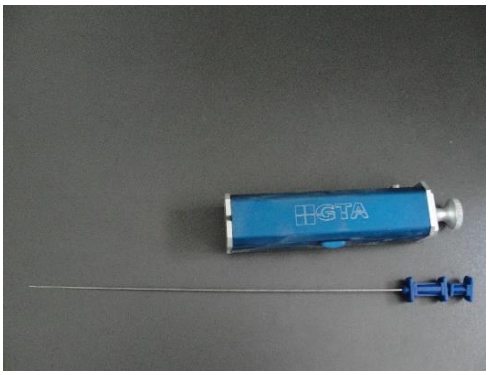

Fig. 1. Automated GTA biopsy gun for ultrasound guided transcutaneous biopsies. Biopsy needle.

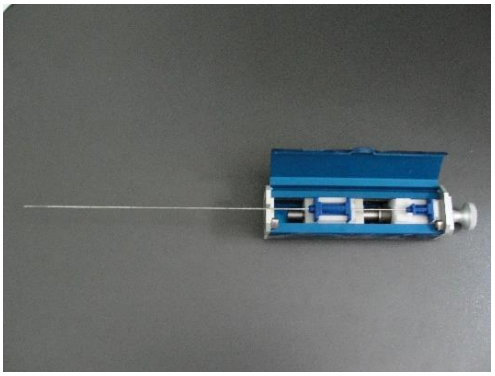

Fig. 2. Armed biopsy gun.

Complications and risks associated with a renal biopsy

The results of the clinical studies indicate that complications of kidney biopsies occur with a frequency of approximately 1% to 18%. This variation in the incidence of complications is directly linked to the health status of the patient at the moment of the biopsy.

The higher incidence of post-biopsy complications was observed in dogs of ages above 4-5 years and weighing less than 4 kg, with a high level of creatinine (above 5 mg/dl). Also, complications sometimes followed general anesthesia in dogs with severe renal insufficiency.

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CYTOPATHOLOGICAL DIAGNOSTIC IN CANINE LYMPHOMA

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Abstract
Lymphomas are tumors that may be frequently diagnosed in dogs. The main suspicion for this neoplasic disease arises when lymphnodes become enlarged without the existence of any obvious antigenic stimulation. Fine needle aspiration from enlarged lymphnodes with the preparation of cytological slides allows for an early and relatively precise diagnostic in lymphoma cases. We used this technique, along with the May Grunwald Giemsa staining method to search for cytological and nuclear abnormalities that might indicate the evolution of a lymphoma in dogs that presented with enlarged lymphnodes. We found a constant mixture of medium sized and large lymphocytes, multinucleated cells, anisokaryosis, various nuclear shapes (multiple indentations), multiple visible nucleoli, variable cytoplasmic/nuclear ratios and cytoplasmatic chromatic properties (basophilia, light basophilia). Also, the mitotic index measured per 5 HPF fields (x400) was sometimes a strong indicative of malignancy. All of the cases diagnosed with lymphoma based on cytopathological examination were later confirmed through histopathological examination. This suggests that this simple and fast technique that is applicable also in small animal practices may be used to diagnose lymphomas in dogs with a relative high accuracy.
Keywords: *cytopathology, lymphoma, dog, cat*

Introduction

Lymphoma is a malignant tumor of the lymphatic tissue, that may manifest in various forms and degrees of malignity. It is a disease that usually may be diagnosed only after a histopathological examination of tissue samples from the affected organs (lymphnodes, liver, intestine, skin). However, in private practices the access to such diagnostic methods may be limited and the time aspect may be of great importance when considering tumor disemination, metastasis, organ failure or the surviral time of the patient (Grant, 2016).

Cytopathological diagnosis on the other hand may provide a compromise between the accuracy of the diagnosis, the complexity of the technique, the cost and time needed to reach a diagnosis and the level of invasiveness (Ressel, 2018).

The cytological features in lymphoma, as described by the literature, include an increased proportion of medium (nuclei 2-2,5 times the size of red blood cell) and large (nuclei 3 times the size of a red blood cell or larger), immature lymphocytes (above 20%), with large and lightly pigmented nuclei and a higher amount of lightly basophilic cytoplasm than the one normally seen in mature lymphocytes. Also, an increased rate of anisokaryosis, anisocytosis, indented nuclei, visible, multiple or indented nucleoli constitute strong indicatives for lymphoma. The mitotic rate is calculated in cytopathology for 5 random high power fields (x400) and expressed as follows: low when zero or one mitotic figure has been noticed, moderate for 2-3 mitosis and high is the count is above 3 (Ressel, 2018; Meuten, 2017; Raskin, 2016).

Material and methods

Fine needle aspiration from enlarged lymphnodes with the preparation of cytological slides allows for an early and relatively precise diagnostic in lymphoma cases. We used this technique, along with the May Grunwald Giemsa staining method to search for cytological and nuclear abnormalities that might indicate the evolution of a lymphoma in dogs that presented with enlarged lymphnodes (Raskin, 2016, Dunn, 2014).

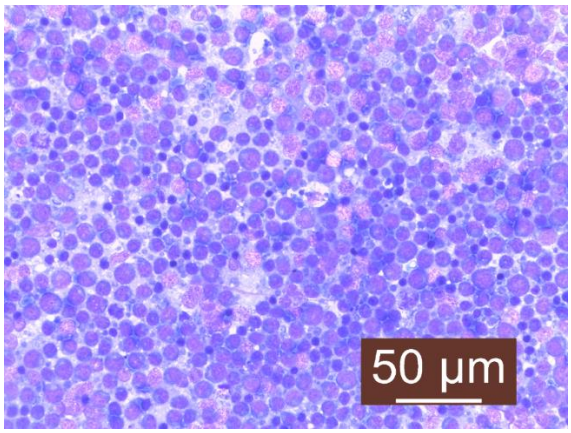
The lymphnodes were punctured using a 23 G needle attached to a seringe. Aspiration was done whilst moving the needle with a fan-like motion, Negative presure was released before

retracting the needle and a new seringe filled with air was reattached to it. The content of the needle was ejected on a slide and lightly compressed between that and another glass slide. MGG staining method followed the standard protocol: 3 min for May Grunwald, 3 min distilled water and 25 min for Giemsa solution (Dunn, 2014).

Examination of the slides was done using a Leica DM 750 optical microscope.

Results and discussion

In all the examined slides we found a constant mixture of medium sized and large lymphocytes (Fig. 1, 2), indicating a continuous process of formation of young, immature lymphocytes with abnormal characteristics. Among these we noticed anisokaryosis, various chromatin patterns (Fig. 3), various nuclear shapes (sometimes with multiple indentations), multiple visible nucleoli (sometimes indentated), variable cytoplasmic/nuclear ratios and cytoplasmatic chromatic properties (basophilia, light basophilia).



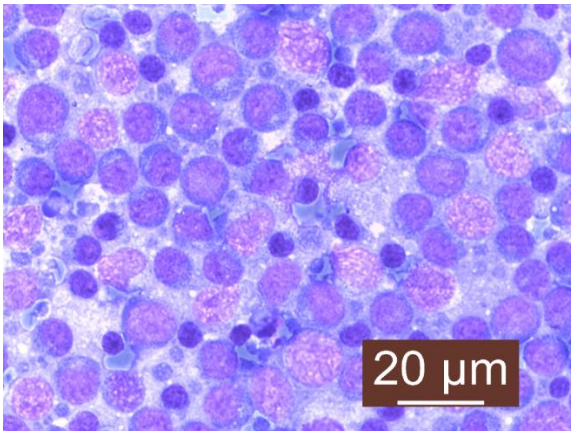


Fig. 1, 2 A mixture of small, medium and large lymphocytes may se seen, with both nuclei and cytoplasm dysplaing various aspects. Lymphoma. FNA from lymphnode. Dog. Masson trichrome stain

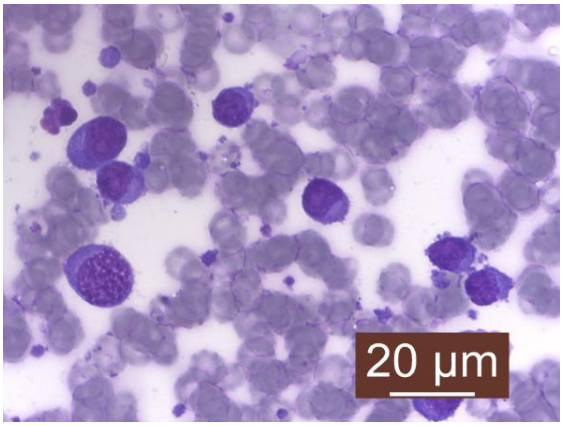


Fig. 3 Stappled cromatin and anisokaryosis. Lymphoma. FNA from lymphnode. Dog. Masson trichrome stain

Sometimes, abnormal cells with multiple nuclei could be observed, the nuclei being of the same or of different sizes (Fig. 4, 5). The cromatin pattern varied between the nuclei of different cells or the ones of the same cell, being either granular, stappled or condensed.

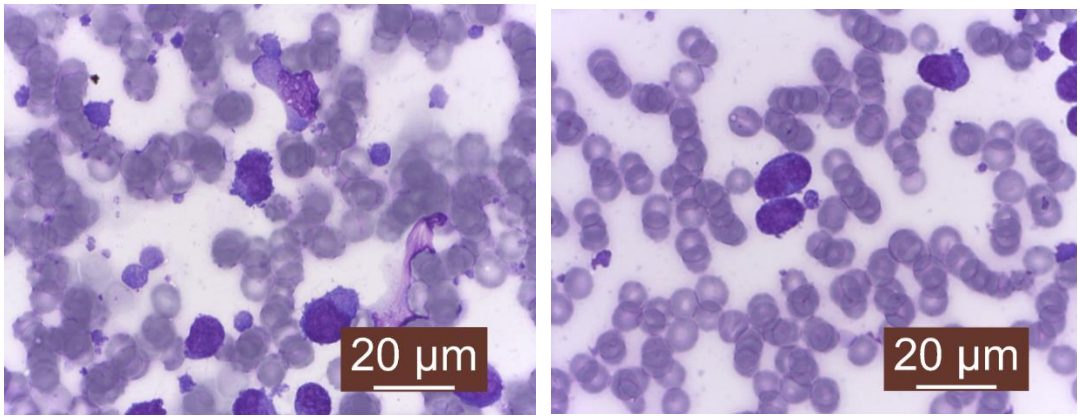
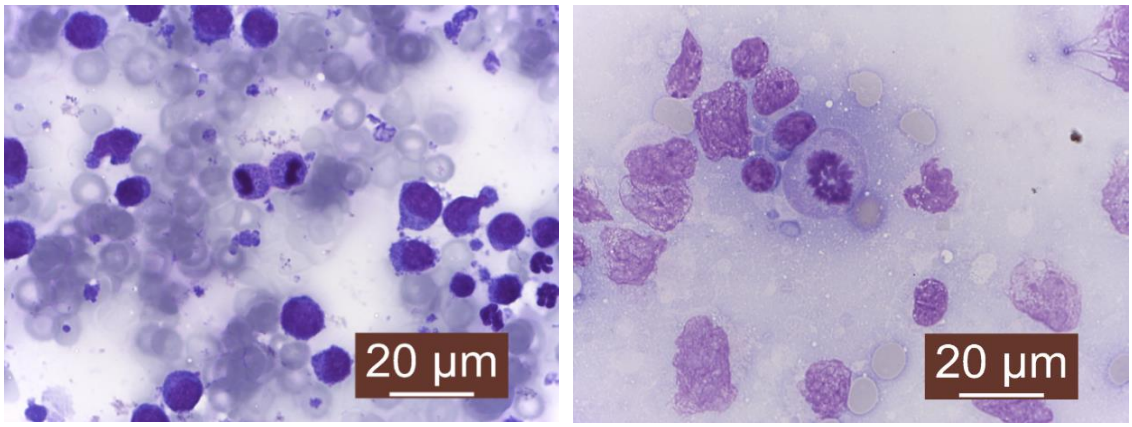


Fig. 4, 5 Multinucleated lymphocytes are also a malignancy criteria. Lymphoma. FNA from lymphnode. Dog. Masson trichrome stain

Also, the mitotic index measured per 5 HPF fields (x400) was sometimes a strong indicative of malignancy. We were able to capture different phases of the mitosis and even found high power fields with 5 mitotic figures, indicating an active proliferative process (Fig. 6-9).



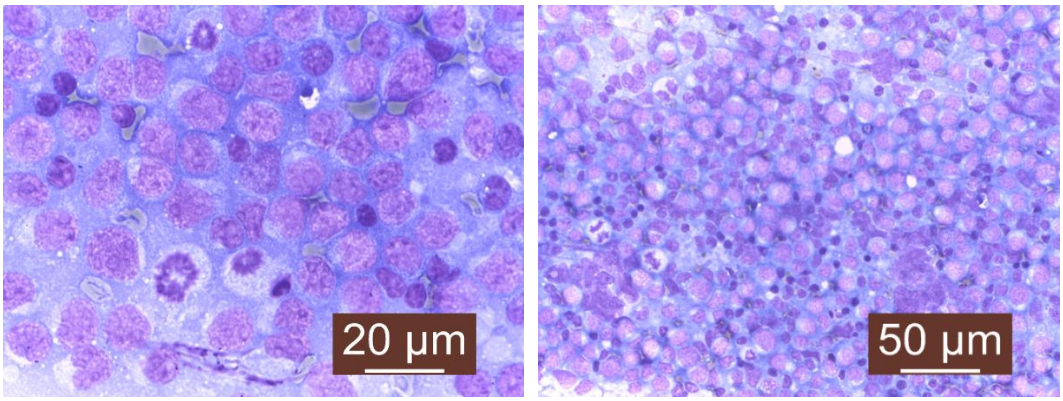


Fig. 6-9 Mitotic figure, both normal and abnormal, sometimes found in high numbers in a single high power field. Lymphoma. FNA from lymphnode. Dog. Masson trichrome stain

All the cases diagnosed with lymphoma based on cytopathological examination were later confirmed through histopathological examination. These results suggest that this simple, unexpensive and fast technique that is applicable also in small animal practices may be used to diagnose lymphomas in dogs.

The widespread of this procedure could help in establishing an earlier diagnostic in lymphomas in dogs, in formulating a more acurate prognostic and in initiating a treatment plan that may help prolongue the life of the patient or prevent metastasis.

Conclusion

Cytopathological diagnosis is a viable option in establishing a diagnosis with relatively high accuracy in canine lymphoma, in lack of or while waiting for the posibility of perfoming techniques and exams that can provide for a more accurate diagnosis.

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A CASE OF MIXED GRANULOMATOUS INFLAMMATION IN A TURKEY HEN

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Abstract
Granulomatous inflammation may be caused in birds either by various infectious diseases (with the formation of Langhans type giant cells) or by the presence in different tissues and organs of foreign bodies (with the formation of Muller type giant cells), the most common being uric acid crystals in gout. We examined the cadaver of a two year old turkey hen that had multiple yellowish-white nodular formations located in the liver. Histopathological examination showed that these were formed mainly through an infectious type granulomatous inflammation caused by a chronic colibacillosis with the presence of bacterial colonies and necrotic debris in the center and Langhans giant cells with foamy cytoplasm. The liver also had early stage foreign body granulomas caused by the precipitation of uric acid crystals in the parenchyma. We were then able to establish that the bird suffered from both coligranulomatosis and also visceral gout, as a consequence of improper microclimate and feeding parameters. It is uncommon to find both types of granulomatous inflammations in the same patient or even in the same organ. The tophy gout had not yet started to form Muller type giant cells, but still, the architecture of the granulomas was clear enough to be compared to the adiacent infectious type one.
Key words: *chronic colibacillosis, gout, granulomatous inflammation*

Introduction

Granulomatous diseases in poultry may be caused by multiple and various factors. First of all, granulomatous inflammation may evolve as a reaction of the immune system towards an infectious agent or a foreign body. Amongst the first there are mostly bacteria (*Salmonella pullorum, Mycobacterium tuberculosis, Escherichia colli, Pasteurella multocida, Staphylococcus aureus, Streptococcus spp., Eubacterium tortuosum)* and parasites *(Tetratrichomonas gallinarum, Histomonas meleagridis,*) that may cause this type of lesion, but also some species of fungi *(Aspergillus fumigatus*) (Landman, 2017; Supartika, 2006; Paul, 2005).

Coligranulomatosis (also known as Hjarre's disease) appears when the immune system cannot eliminate the bacteria and so it chooses to encapsulate it in a focal lesion that is separated from the normal, healthy tissue through several layers of different cell populations. Granulomas may develop in the intestinal tract and liver. A histological characteristic of the coligranuloma is the particular aspect of the giant cells found in direct contact with the bacterial colony and the caseous necrotic debris. They are elongated cells, with a foamy cytoplasm and the nuclei located opposite from the center of the lesion (Zachary, 2017; Paul, 2005; Coțofan, 1992;).

The foreign body granulomatous type reaction is mostly caused by visceral gout, a dysmetabolic syndrome caused by the accumulation of urate crystals in various tissues, triggering a proliferative mesenchymal reaction. The main histological characteristic of this type of lesion is the aspect of the giant cells (hundreds of nuclei located opposite from the foreign body, irregular shape that follows the contour of the foreign body) and their display as a rosette around the urate deposit, in order to minimise the irritative action on the healthy tissue (Paul, 2001).

Material and methods

We received the cadaver of a turkey hen, two years of age, that came from a small farm. The necropsy exam showed multiple yellowish-white nodular formations in the liver. Tissue fragments were harvested and fixated in a 10% formaldehyde solution. After this they were included in paraffin, sectioned at 5 μm and stained following the Masson's trichrome method.

Histopathological slides were observed using a Leica DM 750 optical microscope.

Results and discussion

Histopathological examination showed that the nodular formations were formed mainly through an infectious type granulomatous inflammation caused by a chronic colibacillosis with the presence of bacterial colonies and necrotic debris in the centre of the formations and Langhans giant cells with foamy cytoplasm located on the margins of this area (Fig. 1, 2).

However, the liver also had early stage foreign body granulomas caused by the precipitation of uric acid crystals in the parenchyma (Fig. 3, 4). We were then able to establish that the bird suffered from both coligranulomatosis and visceral gout, the later one being a consequence of improper microclimate and feeding parameters (hyperproteic feed, lacking drinking water, vitamin A deficiency, kidney dysplasia) (Coțofan, 1992).

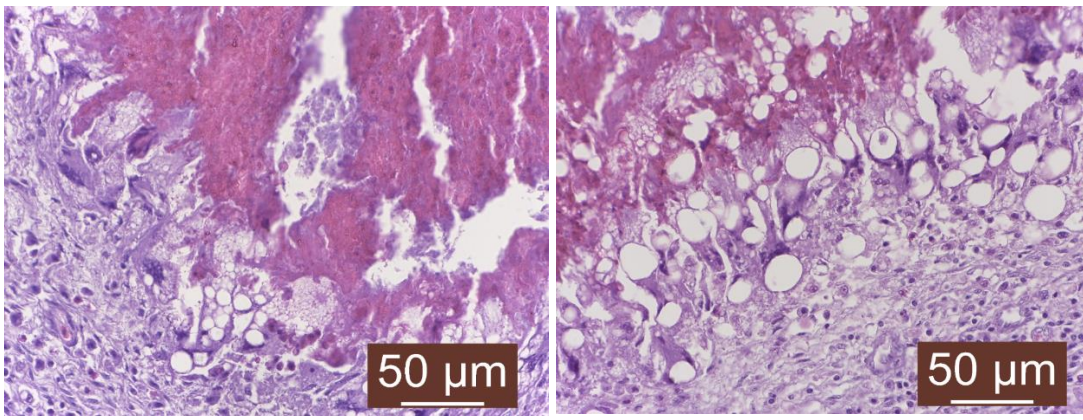

Fig. 1, 2 Coligranuloma in the liver of a turkey hen. Caseous necrotic debris and giant cells with foamy cytoplasm. Masson trichrome stain

It is uncommon to find both types of granulomatous inflammations in the same patient or even in the same organ. The tophy gout had not yet started to induce the formation of mature Muller type giant cells (dark rim of nuclei located opposite from the foreign body), but still, the architecture of the granulomas was clear enough to be compared to the adjacent infectious type one (Landman, 2017; Supartika, 2006).

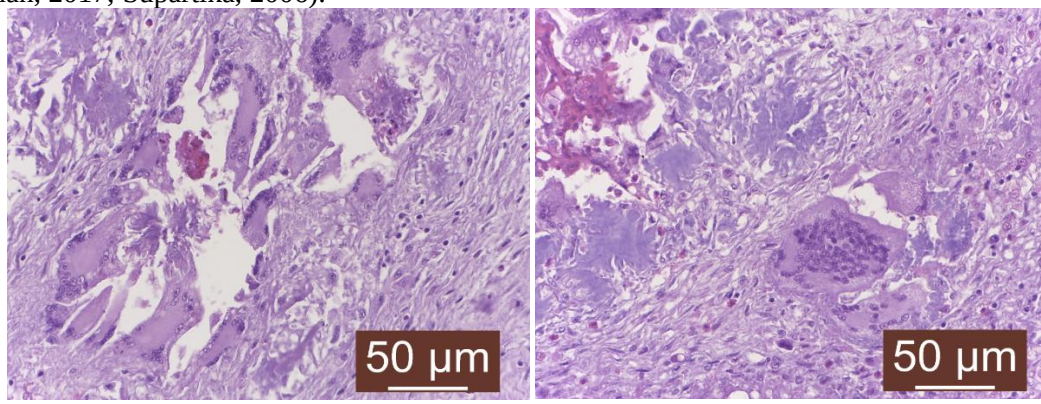

Fig. 3, 4 Tophy gout with young Muller type giant cells forming arround uric acid deposits, adiacent to a coligranuloma. Turkey hen. Masson trichrome stain

The number of the nuclei was much higher and also the aspect of the cytoplasm varied between the two giant cell types. The shape of the cells corresponded with the literature descriptions for these types of lesions, thus facilitating the indentification (Pașca, 2016).

Conclusions

Even though the literature tends to describe lesions in a separate, scholastic manner, it is not uncommon to find many lesions of different types evolving at the same time in the same organ or tissue. In this case, we were able to observe two types of granulomatous inflammation, caused completely different etiological factors, developing within the same macroscopical lesion in the same liver.

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SOME ARGUMENTS FOR AND AGAINST ANIMAL EXPERIMENTATION -A LITERATURE REVIEW-

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Abstract
Preclinical tests on animals are mandatory when new biomedical products are launched on the market, all of these having the main goal to improve the lives of humans, animals or to improve the environment. In this sense, it is necessary to identify or to create the "animal model" that is as close as possible to the human physiology and psychology, taking in account that each species has its peculiarities that make it difficult or sometimes impossible to extrapolate the results from animal to human. However, some of human diseases have been cured just through research on animals so, this paper is bringing for and against arguments regarding animal experimentation. On the other hand, poor communication of research results, minimization of harm in favor of glorious benefits, underestimation of issues of experimental ethics are some of the most claimed subjects by people and researchers.
Keywords: *animal research, arguments, drogs, experimentation on animals*

Introducere

From ancient times until now, experimentation on animals or people has been carried out by methods more or less accepted by the scientific, juridical or religious communities (Gary, 1996; Mullin, 1999; Machado et al, 2017) experimentation on humans being generally restricted to non-life-threatening researches, and the people being voluntary included. Experiments on large masses of population, both human and animal, are prohibited after nowadays legislation..

Data about the first biological experiment came from 450 BC, when Alcmeon de Corotona demonstrated the direct relationship between the optic nerve and the blinding caused by sectioning this nerve in an animal (Boada Saña et al, 2011).

A thousand years ago (11th century AD), Ibn Sina proposed the need of human experimentation (Nasser et al, 2007), N. Shanks and C. Ray Greek (2009) concluded in their book "Animal Models in Light of Evolution" that while animals can successfully be used in many areas of biomedical sciences, such as basic and comparative research, they cannot provide security in anticipating people's response to drugs or diseases (Lubon, 1998; Mullin, 1999; DiMasi et al, 2003; van der Worp et al, 2004; Gawrylesky, 2009; Ormandy & Schuppli, 2014). However, the progress of biomedical sciences, diagnosis, therapy and prevention is undeniable (Mathews, 2008; Shanks & Greek, 2009; Adams, 2010; Bottini & Hantung, 2010).

Organizations who fight for animal rights around the world make relevant arguments against the use of animals in experiments, based on the high failure rate of animal-tested therapies for human treatment (Mathews, 2008; Gawrylewski, 2009; van der Worp et al, 2010; Ormandy & Schuppli, 2014).

Thus, despite the use of over 115 million animals in reasearch worldwide, yearly (Taylor et al, 2008), only 46 new drugs were approved for consumption in 2017 by the US Medicines Regulatory Authority. Food and Drug Administration), many of them are also for treatment of rare diseases (https://www.crueltyfreeinternational.org/why-we-do-it/arguments-against-animal-testing).

It is generally accepted that the development of new drugs is a complex, long and an expensive activity. The cost of producing a drug before it is marketed is estimated at $ 802 million to $ 1 billion in the United States (DiMasi et al, 2003; Adams & Brantner, 2010), and the average time to develop a new drug is about 9 years (Oates, 2006).

A. Harding showed that in 2004, the FDA estimated that 92 percent of drugs that pass the preclinical tests, including "pivotal" animal tests, fail to proceed to market and the failure rate is rather closer to 96 percent (Akhtar, 2015). The estimated causes of failure are determined by the lack of effectiveness and safety problems that were not predicted, avoided or eliminated by using animals (Bottini & Hartung, 2009). The human organism often varies dramatically from the animal with respect to uptake, distribution and excretion of substances, and forms very different metabolites of the same substance (Odom et al, 2007; Berkowitz, 2009; Hantung, 2017).

The stagnation in development of new types of medicines is evident, despite the increase of the research costs compared to previous decades, being evident that only 6% from 4,300 international companies involved in drug development have registered a new drug since 1950 until now (Woodcock & Woosley, 2008).

Through artificializing of growing conditions by changing the habitat, changing the food natural diversity and type and through eliminating the competition for survival and reproduction, all of these lead to alterations of the psychism and physiology of the animal research. Thus, an increase in stress (Akhtar et al, 2008) and changes in behavior or other were registered, showing that the conditions in the laboratory cause changes in neurochemistry and nerve regeneration, genetic expression, or the decline of some animal strains (Odom et al, 2007).

The study of human diseases on animals firstly requires to reproduction of predisposing causes and condition which do not occur in wild animals. The inability to produce the disease state in animals mirroring the human disease-state has led to the failure of more than 114 potential therapies tested on animals (Sena et al, 2010). Also, wild animals, together with conventional reaserch animals do not develop some of the human diseases, such as Parkinson's disease, major types of the heart disease, some types of cancer, schizophrenia, amyotrophic lateral sclerosis, lesions traumatic brain injury, Alzheimer's disease or some inflammatory maladies (Curry, 2006; Lane & Dunnett, 2008; Akhatar, 2015). So, in 2007 it was reported that some drugs proposed for treating Parkinson's, CEP-1347 (Parkinson's Study Group, 2007) and Cogane had failed in human clinical studies after being successfully tested on animals (http://www.pdf.org/ en / science_news / release / pr_1361290946).

A study conducted between 2002 and 2012 which analysed how 244 compounds out of 413 clinical trials about new therapies for Alzheimer's disease showed that from the 244 compounds, only one was approved for human treatment (Cummings et al, 2014). A recent example is the drug Dimebon, which had not been shown to be effective in humans and has been withdrawn (Bezprozvanny, 2010), even though it had passed the animal testing (Lermontova et al, 2000).

Despite successful preclinical tests, about 85% of early clinical studies for the implementation of new anticancer drugs fail, and of those that reach phase III, only half of them are approved for clinical tests (Ledford, 2011).

Although anticancer vaccines have been effective for induceing the immune response in animal models, they have produced mixed (yet inconclusive) results in human clinical tests. Ogi and Aruga (2013) reported in their article that of the 23 phase II/III clinical trials which tested 17 distinct cancer vaccines, 18 of these experiments failed.

The unhappy incident produced in the phase 1 of a study on *the monoclonal antibody TGN 1412* demonstrated the inaccurate prediction of results in humans, even though they had favorable results on nonhuman primates. Some volunteers who tested the monoclonal antibodies at Northwick Park Hospital (UK in 2006) suffered from a severe allergenic reaction. However, the monkeys testing a dose 500 times higher than the dose given to the volunteers failed to anticipate the harmful effects (Stebbings et al, 2007).

In the study of stroke prevention, approximately 500 neuroprotective therapies that were considered successful subsequently failed in humans, (O'Collins et al, 2006; Sena et al, 2010). Out of the 1,000 or more potential drugs for treating or preventing strokes that have been developed through animal testing, only about 10% have been effective in humans (O'Collins et al, 2006; Sena et al, 2010).

According to a study by Shaoni Bhattacharya, it showed that Vioxx (Rofecoxib), a drug designed to treat arthritis, which was considered safe when it was tested on monkeys (and five other species), caused about 140,000 deaths worldwide due to heart attacks and strokes (https://www.newscientist.com/article/dn6918-up-to-140000-heart-attacks-linked-to-vioxx/). After this sinister event it was been withdrawn from the market in 2004.

Some of these examples about the failures of some products tested on animals and used in human therapy overshadow the remarkable achievements of biomedical research but at the same time force the researchers, animal welfare organizations, pharmaceutical concerns and equipment production factories to progress, to allocate more funds in order to create new opportunities for experimentation, according to the 3Rs principle or ethical and legal reasons.

It is known that the testing the safety of medical devices, such as cardiac valves, stents in animals (Rivard et al, 2007) or animal intubation protocols (Kircher et al, 2009) before they are introduced in human clinical trials requires the analysis of the risk during the preclinical studies (Fisher et al, 2009) making more accurate determinations of the risk for each device in the matter of compatibility with the morphological structures and the short and longtime influence of the physiological parameters, etc of the body. Also, the animals are used for testing the dental and bone implants (Natiella, 1988) or for developing of performing prosthetics (Arias et al, 2013) etc.

Refining of the growing conditions of laboratory animals through obtaining the germfree animals, but especially through producing the animal models (transgenic animals) after genetic manipulation to express the genes of interest (which are thought to involve the onset of pathological conditions) being used to study some physiopathologies of human or animal diseases, these represent the truly achievements in medical research (Maga et al, 2003). Thus, the experimental models were obtained to study some types of human cancer [eg: Oncomice which overexpress the oncogenes (Hanahan et al, 2007), pig which develops the colonectal cancer (Flisikowska et al, 2012), etc], experimental models for some genetic diseases such as retinitis pigmentosa (Banin et al, 1999); experimental models for the study of atherosclerosis (Rennert et al, 2008; Perleberg et al, 2018), in studies in the matter of antiading, improving memory and learning in that are used, fo example the Doogie mice (Tang et al, 2001; Flinn, 2016), etc.

Also, through genetic engineering were obtained the lactose-free cow's (goat's) milk (Karatzas & Turner, 1997; Kabotyanski et al, 2009), or milk with a similar composition to human milk (Yang et al, 2008; Kabotyanski et al, 2009), secretion of some antiviral antibodies by milk (Young et al, 1998) or some blood clotting factors (Lubon, 1998; Lindsay et al, 2004; Van Cott et al, 2001).

An other exemple is the reduction of environmental pollution caused by high phosphorus levels of pig faeces which was solved by producing transgenic pigs whose genome contained an inserted gene encoding *phytase* of bacterial origin, so that the phosphate level in the faeces of transgenic pigs decreased by 75% (Golovan et al, 2011).

Conclusion

Animal experimentation has proven to be an extremely effective tool for biomedical research, and it is inconceivable to use products without animal testing during the preclinical studies. However, the arguments against the use of animals in research represent in fact the source

of progress, any failure will lead to improve the intra-experimental protocols and to selection of that animal model which is the most faithful to the targeted goal.

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IMPACT OF *STAPHYLOCOCCUS AUREUS* AND METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) ON UTERINE DISEASE IN DAIRY CATTLE AFTER PARTURITION

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Abstract
The aim of this study was to identify vaginal flora in Black and White Romanian cows diagnosed with puerperal endometritis. The cows studied came from two dairy farms, were in the first 4 weeks post-partum and had various puerperal diseases with variable pathological evolution. The dinamics of isolation of different bacterial species from lochia or vaginal discharges was made in four periods of the puerperium. Bacteriological examination was performed in accordance with routine laboratory techniques, including typical colony morphology on usuale culture media, chromogenic agar, Gram stain, type of hemolysis, characteristic growth on Baird-Parker (BP) agar and biochemical tests. In the course of puerperium, approximately 22,2% of microorganisms detected in the 18 cases were represented by the Staphylococcus aureus, what means that may be a significant pathogen of puerperal infection. Isolation in 11,1% of cases of methicillin-resistant stains of Staphylococcus aureus (MRSA) is important in terms of direct impact on human and animal health.
Key words*: Cows, Puerperal endometritis, Staphylococcus aureus, MRSA*

Introduction

A large number of microorganisms have been reported to contaminate the uterus of dairy cows after calving, and these microorganisms are recognised as a major etiological factor of uterine disease (Földi and others 2006). After parturition, bacteria from the animal's environment contaminate the uterine lumen of most cattle.

Uterine infection can be categorized into puerperal metritis, clinical metritis, clinical endometritis, and subclinical endometritis (Sheldon et al., 2006).

Endometritis is one of the most common diseases that occurs in dairy cow after several weeks postpartum period (Sheldon et al., 2006).

In the context of clinical endometritis, staphylococci are generally considered to be potential pathogens or opportunist contaminants (Williams E. et al., 2005).

Staphylococcus aureus (*S. aureus*) is a microorganism that is present as a commensal on the skin, the nose and mucous membranes of healthy humans and animals (Turner N.A. et al., 2019).

In recent years, studies into livestock-associated *S. aureus* including methicillin-resistant (MRSA) strains have provided new information regarding their origin and host adaptation, and their capacity to cause zoonotic infections of humans (Ross Fitzgerald J., 2012).

MRSA in animals

The presence of methicillin-resistant Staphylococcus aureus (MRSA) in animals such dairy cattle involve a probable human origin adapted to cattle, but also was found distinct bovine types which are clearly different from human isolates (Cuny C. et al, 2010).

MRSA was first detected in the early 1970s from the milk of dairy cows with mastitis in Belgium, and these samples were most likely contaminated by humans (Devreiese, 1975).

After Belgium, other geographic areas had reports of MRSA frequently in livestock animals, dairy cattle and milk (Vanderhaeghen W, 2010).

Since the raport from 1972, MRSA was found in other domestic species, like dogs (Pak et al., 1999, Loeffler et al., 2005), cats (Bender et al., 2005), horses (Anzai et al., 1996, Hartmann et al.,1997), sheep (Goni et al., 2004, Gharsa et al., 2012) and pigs (Voss et al., 2005).

MRSA strains from genital samples have been identified in lemurs, chimpanzees and gorillas in Africa (Lozano C. et al., 2015).

Anzai T. et all, (1996) isolated methicillin-resistant *Staphylococcus aureus* strains from mares with metritis and the infection was from a stallion with skin lesions of the hind leg.

The majority of the studies suggested that the samples with MRSA found in dairy cattle were contaminated by humans, but some reports indicated that the MRSA present in cows is bovine-specific (Lee J.H., 2003).

Material and method

Criteria for inclusion in the study:

- the bacteriological examination was performed on samples taken from cows in the first four week after parturition;
- 18 Black and White Romanian dairy cows from 2 farms (Iași County) with clinical signs of clinical endometritis that persist more than 21 days after calving;
- the diagnosis of endometritis on clinical examination and the presence of purulent vaginal discharge within 21 days or more after parturition (Sheldon and others, 2006);
- purulent secretions were recovered from cows without antibiotic treatment in history.

To perform this examination, lochia and genital secretions were collected in sterile conditions, from the vulvar, vaginal or uterine level.

Isolation of Staphylococcus aureus strains

The samples were collected in sterile swabs, and were trasported in a short time and in refrigeration conditions. After collecting the discharges were immediately inoculated into liquid culture medium and incubated at 37ºC for 24 h aerobically.

The isolation and direct identification of *Staphylococcus aureus* and *Methicilin Resistant Staphylococcus aureus* was carried out on chromogenic agar plate, Sa Select and MRSA Select II Medium from Bio-Rad Laboratories (fig.1 and fig.2).

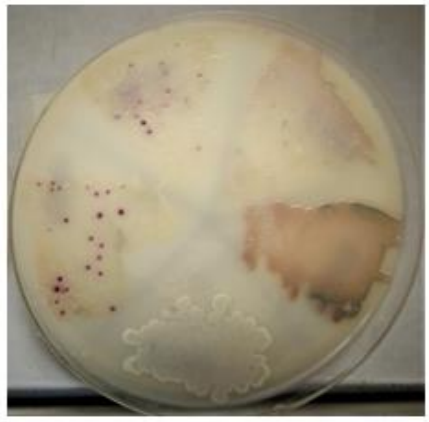


Fig.1 Pink colonies of *Staphylococcus aureus* on SaSelect Agar

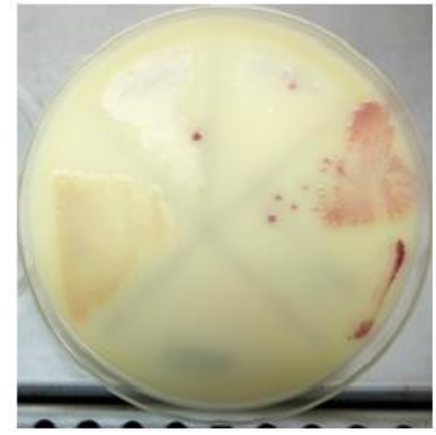


Fig. 2 Pink colonies of MRSA on MRSA Select Agar

To confirm the identification of these bacteria have beed made additional testing.

For each sample were isolated and selected typical *S. aureus* and MRSA colonies for purification (24-48h at 35ºC) and further characterization on non-selective medium (blood agar and Muller Hinton Agar, Bio-Rad Laboratories).

Staphylococcal colonies (orange to pink colonies of *S. aureus* from SaSelect Agar and pink colonies from MRSA Select Agar) were inoculated on blood agar (that contains 5% sheep red blood cells) to observ beta-hemolysis produced by *Staphylococcus aureus* (fig.3).

Fig.3 *Staphylococcus aureus*. Beta hemolysis on blood agar.

Staphylococcus aureus is characterized by a round shape (coccus or spheroid shaped), Gram-positive (purple), and found as either single cells, in pairs, or more frequently, in clusters that resemble a bunch of grapes (fig.4).

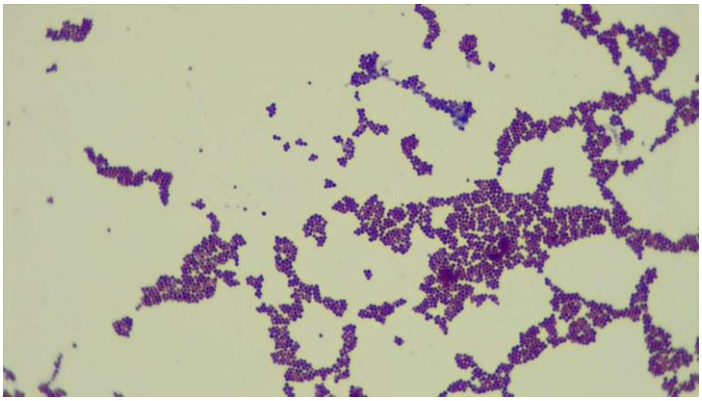


Fig.4. Methicilin resistant *Staphylococcus aureus* cells (Gram positive coci), Gram staining, x1000 MO

The presence of methicillin resistant *Staphylococccus aureus* (MRSA) in clinical samples were evaluated in two chromogenic agar plates, MRSA Select II and Brilliance.

On Brilliance agar the typical colonies of MRSA were denim-blue (fig.5). Both culture media are sensitive for screnning MRSA. The detection of MRSA in chromogenic agar media was reported in recent studies performed by van Loo IHM et al. (2007), Haitske Graveland et al. (2009), Riedel Stefan et al.(2010).

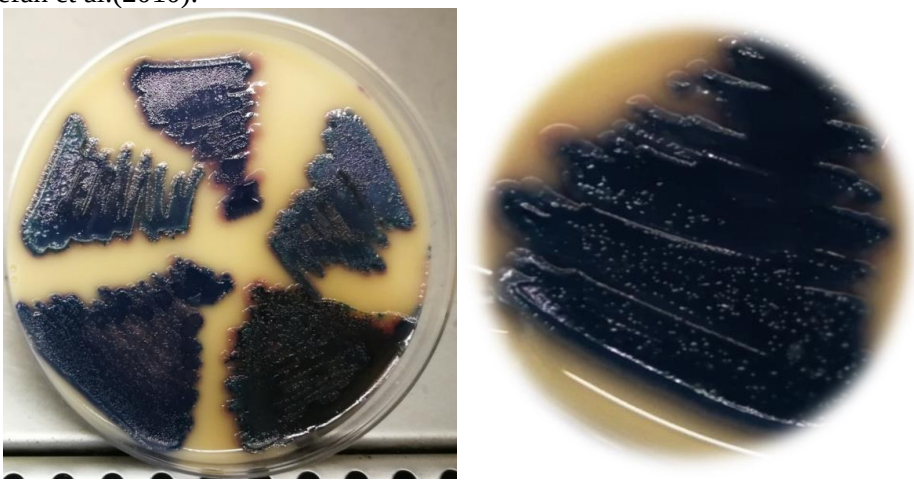


Fig. 5. Denim-blue colonies of MRSA. Brilliance agar

The Baird Parker Agar (Oxoid) allows the growth of *Staphylococcus aureus* and selectively inhibits the growth of other bacteria (fig.6).

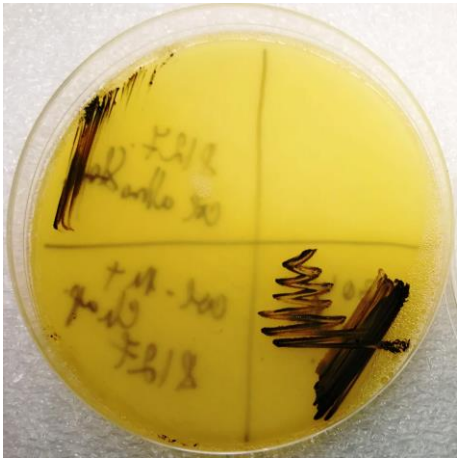


Fig. 6. *Staphylococcus aureus* on Baird-Parker Agar

Further, the biochemical test were performed for confirmation. The identity of *Staphylococccus aureus* isolated on Baird-Parker Agar was confirmed with a coagulase reaction.

Mannitol Salt Agar (Chapman medium) was used for the selective isolation and differentiation of *Staphylococcus aureus* from mixed culture (fig.7).

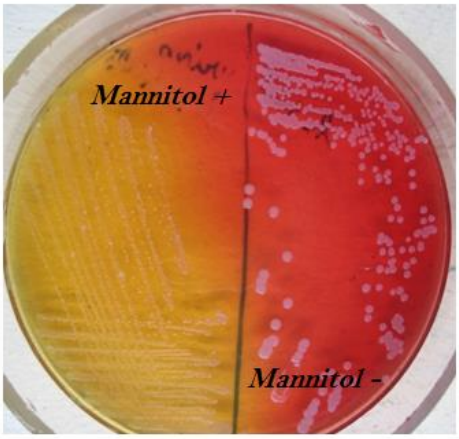
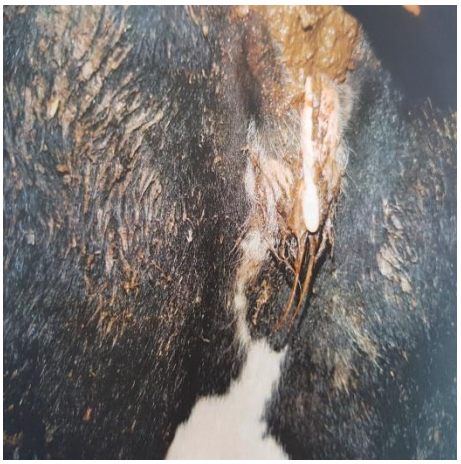


Fig. 7. *Staphylococcus aureus* (Mannitol +), yellow colonies with yellow zones on Chapman medium

Results and Discussion

Clinical signs on puerperal endometritis

Confirmation of clinical endometritis was made based on vaginal discharge during trans-rectal examination. The observed leaks had a sero-mucous, yellowish-brown appearance, sometimes with fibrin deposits.



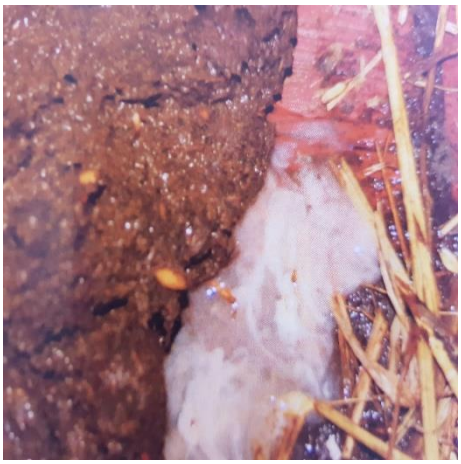


Fig. 8. Clinical appearance of vaginal discharge in purulent endometritis

Bacteriological examination

Out of 18 cows were sampled at 28-55 days postpartum and all contained different strains of bacteria. *Staphylococcus aureus* and *Methicilin resistant Staphylococcus aureus* were detected in 4 (22,2%) and 2 (11,1%) samples in pure culture. MRSA was detected in 2 samples in pure culture from 18 cows.

The 2 tipes of bacteria were identified based on cultural characters from chromogenic agar, Gram stained, selective media and confirmed by standard biochemical tests like -Chapman medium (mannitol +), Baird Parker medium, coagulase test, API STAPH test.

Previous results have suggested that Staphylococcus aureus was a predominant species isolated from cows with puerperal endometritis (Salah N. et al., 2017).

Staphylococcus aureus and *Methicillin Resistant Staphylococcus aureus* have a major implication in healthcare infections worldwide, because of the cattle contact as a risk factor for MRSA colonization in humans (Cunny C. et al., 2010).

Acording to an aetiological investigation of China's dairy cow, *Staphylococcus aureus* (SA) is one of the most common pathogen of endometritis (Meng Dan et al, 2019).

Éva Juhász-Kaszanyitzky et al., (2007) reports an infection rate of Staphylococcus aureus at aprox. 20-50%.

The massive use of antibiotics on farms has led to the adaptation and evolution of pathogens so the cattle can represent ecological niches for the development of multidrug resistance in MRSA strains (Gill S.R., et al., 2005).

Cows can carry S. aureus in their skin, udder, nasal cavity and rectum. S. aureus multiplies and survives in animal organisms, but can live in the environment for a long time, and then the most important route of transmission is by the hands of the farmer, veterinarian or caretaker (Marsilio F., et al., 2018).

Humans are the natural hosts for *S. aureus* and MRSA and the appearance of MRSA in dairy farms is a serios threat to public health because of cross-infection between animals and humans (Stefani S. et al., 2012).

The detection of MRSA isolates cultured from blood and infected wound sites in humans is strong circumstantial evidence that these organisms are capable of causing clinical disease (L.Garcia-Alvarez et al., 2011).

Conclusions

The results suggested that *S. aureus* might play a major role in cow endometritis infection. The discovery of MRSA in dairy cows suggests that these animals might provide a reservoir of infection and close links with farms or contact with dairy cattle could be risk factors that increase the likelihood of MRSA carriage or infection in patients.

Due to the high capacity of *Staphylococcus aureus* to acquire, maintain and mobilize antimicrobial resistance genes, further molecular surveillance is essential to monitor their progress over time.

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MOTILITY PARAMETERS OF EQUINE EPIDIDYMAL SPERMATOZOA AFTER 24 HOURS INTRA-EPIDIDYMAL EXPOSURE TO LIDOCAINE USING TWO COMMERCIAL EXTENDERS

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Abstract

Epididymal spermatozoa is the last source for gamete rescue in case of emergency castration or sudden death of a valuable stallion, thus an ideal harvesting and preservation technique should be employed. Routinely, 2% lidocaine intraparenchymatous administration is used to provide analgesia prior to castration, but studies on the effect of lidocaine on epididymal spermatozoa motility parameters are limited. The purpose of this study was to determine the effects of lidocaine on equine epididymal spermatozoa, after 24 hours intraepididymal cool storage using two commercial extenders. We hypothesized that intraepididymal prolonged exposure to lidocaine, might affect motility parameters of epididymal stallion spermatozoa and that different extenders might have an impact. Sperm was collected from 20 epididymides of routinely castrated 3 year old KWPN stallions. 4 stallions received 10 ml 2% lidocaine intraparenchymatous 10 minutes prior to castration and 6 stallions were not medicated. Testicles were transported to an equipped facility and cooled stored for 24 hours. From each sample an aliquot was diluted in a commercial egg yolk based extender, and another in a commercial extender containing defined milk proteins. Motility parameters were registered 30 minutes after dilution, computer assisted. There were no statistical differences between motility parameters of spermatozoa exposed to lidocaine and spermatozoa not exposed, however progressive motility and linearity significantly differed among the two extenders.

Keywords: *stallion, epididymal spermatozoa, lidocaine, commercial extenders, motility parameters*

Introduction

Regional analgesia by means of intraparenchymal 2% lidocaine administration during surgical procedures such as orchidectomy has proven to be effective in reducing pain during routine castration [1,2] or laparoscopic cryptorchidectomy in horses [3]. In one study, administration of 10 ml intraparenchymal 2% lidocaine did not decrease total motility (TM), progressive motility (PM), velocity of the average path (VAP), velocity of the curved line (VCL), linearity (LIN), normal morphology (M) and membrane integrity (MI) of the spermatozoa in vivo, even though relevant concentrations of lidocaine can be detected in epididymal flush, regardless of the blood barrier [4]. This is important in case of emergency castration of valuable stallions, when cryoconservation of cauda epididymis spermatozoa is the last chance to preserve genetic material. Even though viability of spermatozoa significantly decreases after 72 hours, they can be successfully cryopreserved and maintain their fertilization capacity in vitro, after 96 hours of intraepididymal storage at 4 degrees Celsius [5]. Chosing the best extender is crucial when dealing with epidydymal spermatozoa, due to the extremely limited quantity, and extenders can influence the motility parameters of refrigerated epididymal spermatozoa [6]. To the authors knowledge, there is no other study on the

effect of different commercial semen extenders on epidydymal spermatozoa after intraparenchimatous injection of lidocaine.

The purpose of this study was to determine the effects of lidocaine on equine epididymal spermatozoa, after 24 hours intraepididymal cool storage using two commercial extenders. Because lidocaine does cross the blood barrier into the epididymis of the stallion [4], and facilities equipped with the means to extract equine epididymal spermatozoa might be at a a considerable distance, we conducted this study increasing the exposure of spermatozoa to lidocaine during cooled storage. We hypothesized that intraepididymal prolonged exposure to lidocaine, might affect kinematic parameters of epididymal stallion spermatozoa and that different extenders might have an impact.

Materials and method

Animals and castration method

Ten 3 year old KWPN stallions were routinely castrated using a closed technique with the stallions in dorsal recumbency. After a thorough examination of the testicles to detect any gross modifications, each stallion was sedated using xylazine hydrochloride (1.1 mg/kg IV) and butorphanol tartrate (0.03 mg/kg IV). Anesthesia was induced using ketamine hydrochloride (2.2 mg/kg IV) and diazepam (0.07 mg/kg IV). Following aseptic preparation of the scrotal area 10 ml 2% lidocaine hydrochloride (LidoBel, bela-pfarm) was randomly administered to 4 stallions in the parenchyma of the testes using an 18 g 1.5-inch needle. Each testicle was removed using a transfixic ligature (PGA USP 2, SMI, Surgicryl) and a Reimer's emasculator placed approximately 2 cm above the transfixic ligature. Immediately after the removal of the testicle, a hemostatic forceps was placed over the vas deferens in order to prevent leakage and contamination of spermatozoa. Following removal, each testicle was individually packed in a sterile bag, identified and stored at 5 degrees Celsius for transportation, avoiding direct contact of the epididymis with the ice packs. After castration, the stallions were medicated with tetanus toxoid (6000 UI IM) , flunixin meglumine (1.1 mg/kg IV) and a combination of procaine penicillin and streptomycin sulphate (procaine penicillin 4.000 UI/kg and streptomycin sulphate 15 mg/kg IM).

Sperm collection and analysis

After 24 hours intraepididymal cooled storage at 5 degrees Celsius, each cauda epididymis and vas deferens were carefully removed from the testicles after placing a mosquito forceps at the palpable base of the cauda epididymis and dissected free of blood vessels and connective tissue, using an aseptic technique. Spermatozoa was recovered using a retrograde flush technique as previously described [5].

From each stallion, an aliquot was extended to 20 x 10^6 sperm/ml in an extender for chilled semen containing defined milk proteins (EquiPlus, Minitüb, Tiefenbach, Germany; pH 6.8 ± 0.2, 320 ± 20 mOsm/L), and another aliquot was diluted to 20 x 10^6 sperm/ml in an extender for chilled semen containing egg yolk (Gent, Minitüb; pH 6.6-6.8, 310-330 mOsm/L), randomly. Each sample was assessed after 30 minutes maintenance at room temperature for motility parameters using a computer assisted sperm analysis system (SCA® Production, MICROPTIC). Total motility (TM), progressive motility (PM), velocity of the average path (VAP), velocity of the curved line (VCL) and linearity (LIN) were recorded for each sample. Sperm motility was assessed with Sperm Class Analyzer -SCA (Microoptic, Barcelona, Spain) using the following settings: 10x Nikon, negative phase contrast (PC-) optics, calibrate value 0.82µm/pixel, gird distance: 10 µm, box size: 200pixels, VCL/VAP area 4 µm2/min, area:75 µm2/max, static cells threshold <10 µm/s, slow medium 45 µm/s, rapid >90 µm/s, progressive STR >75, VAP points 5 pixels, connectivity 12 pixels. A total of 500 spermatozoa in minimum four fields were assessed using 20 µm Leja slides.

Statistical method

An unpaired t test was used to compare kinematic parameters of lidocaine exposed and non exposed samples using two different semen extenders (GraphPad Prism 6.0). Significance was assessed at $p < 0.05$.

Results

Motility parameters using Equi **Plus Semen extender**

A number of ten pellets were suspended in Equi Plus Semen extender and distributed in one of the two groups: Equi Plus without lidocaine (EP) (n=6) and Equi Plus with lidocaine (EPL) (n=4). Table 1 provides detailed information about the motility parameters analyzed in both EP and EPL groups and the average results. There were no statistical differences between the two groups (fig 1 A-E).

	TM %	PM %	VCL mm/s	VAP mm/s	LIN %
EP 1	99,86	30,52	64,06	35,59	36,68
EP 2	99,74	44,06	74,10	39,67	32,52
EP 3	98,59	13,47	50,43	22,11	29,52
EP 4	97,78	19,70	52,16	23,91	22,67
EP 5	97,67	11,20	42,96	22,07	30,93
EP 6	73,56	16,09	43,20	26,43	41,81
AV	**94,53**	**22,51**	**54,49**	**28,30**	**32,36**
EPL 1	82,03	16,09	46,81	25,39	29,86
EPL 2	72,36	16,91	49,50	27,23	27,82
EPL 3	99,31	34,52	63,49	37,70	39,84
EPL 4	97,71	12,61	45,61	24,34	29,34
AV	**87,85**	**20,03**	**51,35**	**28,67**	**31,72**

Table 1

Motility parameters for Equi Plus group. EP: Equi Plus without lidocaine, EPL: Equi Plus with lidocaine, AV: Average results. Motility parameters TM: total motility, PM: progressive motility, VCL: Velocity of the average curve, VAP: Velocity of the average path, , LIN: Linearity.

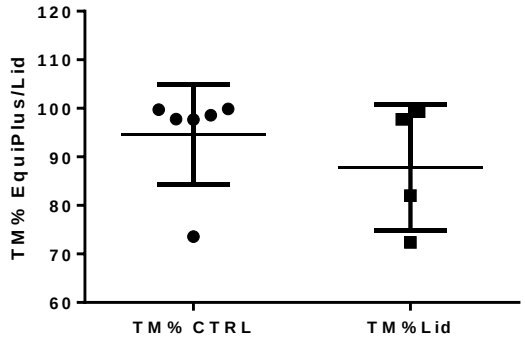

Fig. 1A Total motility for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect TM

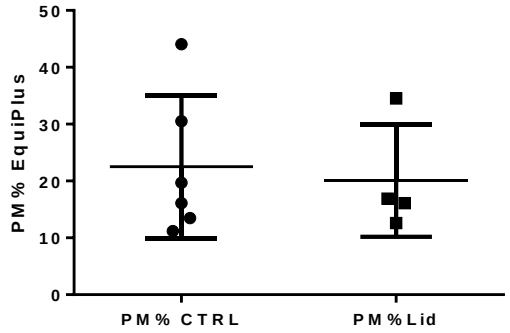

Fig. 1B Progressive motility for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect PM

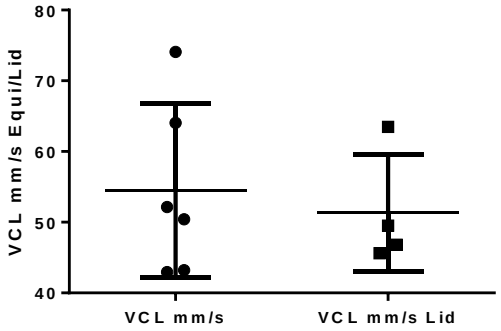

Fig. 1C Velocity of the average curve for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect VCL

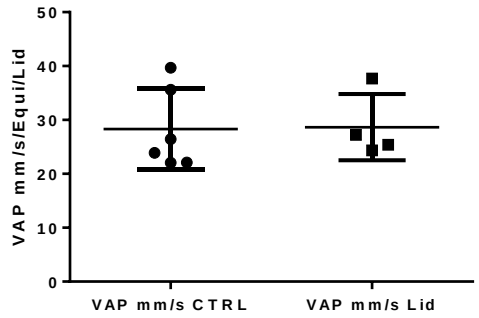

Fig. 1D Velocity of the average path for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect VAP

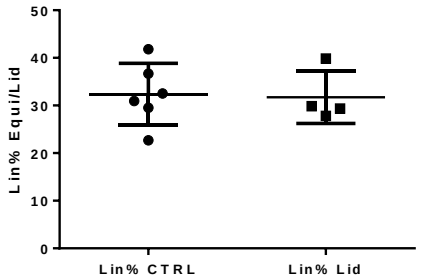

Fig. 1E Linearity for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect Lin

Motility parameters using Gent extender

The remaining samples, extracted from the epididymides of the ipsilateral testicle of the same stallion were suspended in Gent extender and distributed in one of the two groups: Gent without lidocaine (Ge) (n=6) and Gent with lidocaine (GeL) (n=4). Table 2 provides detailed information about the motility parameters analyzed in both Ge and GeL groups and the average results. TM, VCL and VAP showed no statistically significant difference among the two groups (fig 2 A-C) whereas PM and LIN were significantly different among the two groups (fig 2 D, E).

	TM %	PM %	VCL mm/s	VAP mm/s	LIN %
Ge 1	98,54	22,13	52,93	26,60	26,79
Ge 2	99,83	25,36	62,73	30,82	24,63
Ge 3	87,42	7,98	35,90	17,92	25,74
Ge 4	87,35	19,09	49,11	24,71	26,29
Ge 5	99,82	13,82	47,51	30,31	44,01
Ge 6	92,16	8,34	38,11	18,75	26,09
av	**94,19**	**16,12**	**47,72**	**24,85**	**28,93**

GeL 1	85,65	6,61	34,57	17,92	28,00
GeL 2	59,39	15,16	26,68	13,26	26,76
GeL 3	89,29	9,45	35,78	17,80	27,62
GeL 4	96,99	51,81	37,75	20,12	29,90
av	**82,83**	**20,76**	**33,70**	**17,28**	**28,07**

Table 2

Motility parameters for Equi Plus group. EP: Equi Plus without lidocaine, EPL: Equi Plus with lidocaine, AV: Average results. Motility parameters TM: total motility, PM: progressive motility, VCL: Velocity of the average curve, VAP: Velocity of the average path, , LIN: Linearity.

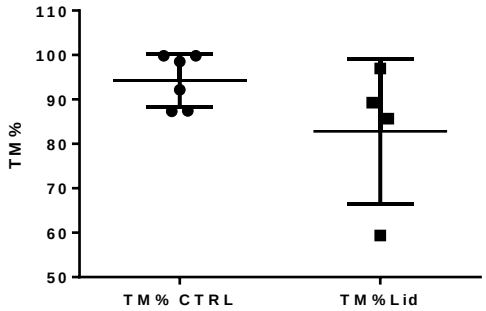

Fig. 2A Total motility for the samples diluted in GENT extender. Lidocaine did not significantly affect TM

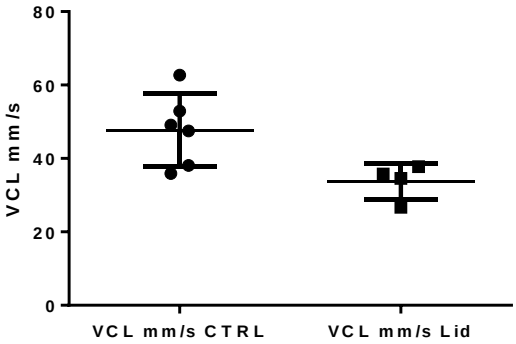

Fig. 2B Velocity of the average curve for the samples diluted with Gent extender. Lidocaine did not significantly affect VCL

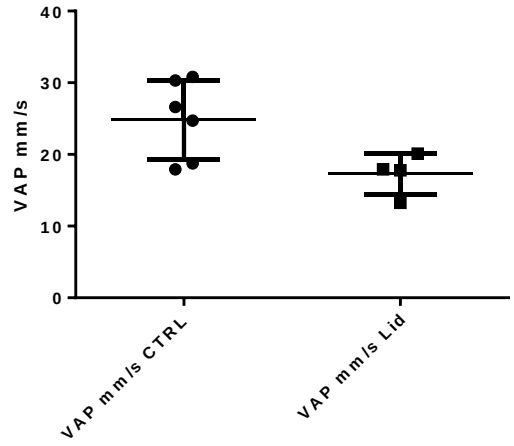

Fig. 2C Velocity of the average path for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect VAP

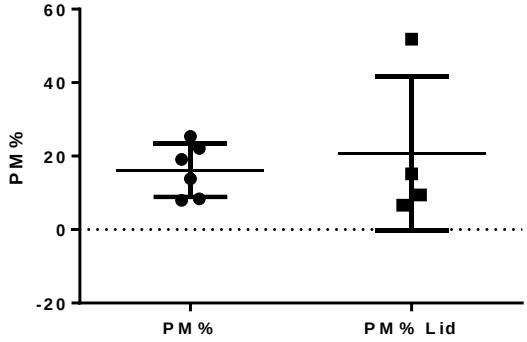

Fig. 2D Progressive motility for the samples diluted in Gent extender. Lidocaine significantly affected PM

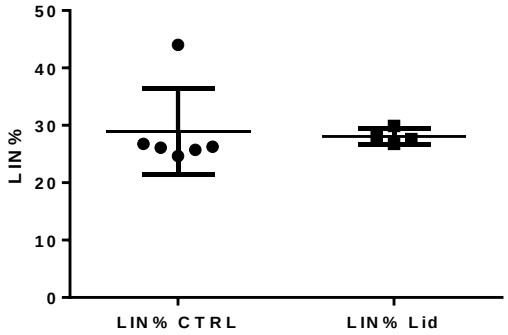

Fig. 2E Linearity for the samples diluted in Gent extender.
Lidocaine significantly affected Lin

Discussion

The present study evaluated motion characteristics of chilled epididymal sperm, after 24 hours intra epididymal exposure to lidocaine, using two commercial extenders. Testing different

semen extenders is not an option with epididymal sperm, due to quantity limitations. Applying a general protocol that works best for a significant number of stallions is the best option. In the current study, the extender for chilled semen containing defined milk proteins produced better results for parameters PM and LIN , compared with the egg yolk extender, in epididymal spermatozoa exposed to lidocaine for 24 hours. This results are similar to other studies, where egg-yolk extenders were proved less efective for both chilled storage of epididymal spermatozoa [7,8], regardless of the lidocaine exposure.

In order to be able to preserve genetic material from deceased or emergency castrated stallions, a method has been described where intraepididymal storage can keep spermatozoa viable for up to 96 hours post castration when epididymides are cooled stored, but motility and most importantly progressive motility decreases time-dependent [9]. This is important when a facility equipped for the procedure is situated at a considerable distance. In the curret study, motility parameters were decreased in all four groups, compared to the Boye study [2], probably due to the prolonged intraepididymal cooled storage.

Lidocaine is routinely used for local analgesia during castration, due to its proven positive effects [10,11]. Similar to another study [2], lidocaine did not seem to affect motility parameters of epididymal spermatozoa even after 24 hours intra-epididymal exposure. The concerns about lidocaine local analgesia were based on the lack of studies regarding the effect of equine spermatozoa exposure to lidocaine, even though results published on human sperm motility showed no negative impact of lidocaine on human spermatozoa, at different concentrations, in vitro [12]. Furthermore, studies on male humans and stallions prove that lidocaine does cross epididymal barrier after being administred in the testicular parenchyma [5, 12].

In the current study, progressive motility and linearity were lower for the samples diluted in Gent chilled extender, regardless of the exposure to lidocaine. Prediction of the fertility of equine sperm prior to cryoconservation remains challenging, and even though latest studies suggest the use of more than one technique in assesing fertility [13], progressive motility is still an important parameter, used to determine the minimum standard requirements for semen for artificial insemination [14] .Interstingly, in the Boye 2019 study, high concentrations of lidocaine did affect the progressive motility and linearity of spermatozoa.

Conclusion

The administration of 10 ml 2% lidocaine intraparenchymatous during routine castration, did not negatively affect motility of epididymal spermatozoa after 24 hours intraepididymal cooled storage, when milk protein chilled semen extender was used. However, when egg-yolk chilled semen extender was used, progressive motility and linearity significantly decreased.

Based on this findings, the injection of lidocaine during routine castration can be used even when epididymal sperm is to be retrieved and milk based extenders seem to improve kinematic parameters of epididymal spermatozoa.

Disclosures

All authors have no conflict of interest to disclosure.

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ADAPTIVE BIOPLASTIC DISORDERS OF THE KIDNEY IN DOGS – REVIEW

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Within this category of kidney lesions, we will talk about tissular growth disorders and topographical anomalies.

Most of these lesions are hereditary and congenital, but they can also be acquired during the life of the animal.

Hypoplasia, the most frequent renal dysplasia, represents the underdevelopment of the organ during its period of growth. The lesion is described especially in very young dogs, with the term of immature or fetal kidney and can be uni- or bilateral.

Macroscopically, the kidneys are smaller than normal, firm, pale and with a wrinkly, pseudo-lobulated aspect. The underdevelopment of the kidneys may be accompanied by the morphofunctional immaturity of the liver and thymus, the later being identifiable upon microscopical examination. In the unilateral form, usually affecting the left side, the kidney appears small, firm, with a thick, adherent capsule, and on the sectioned surface we may notice striations located in the cortical corresponding to fibrous tissue and hypoplasic interlobular areas.

Microscopically, in the cortex, we will be able to observe a reduction in the number of glomeruli and tubular hypertrophy. In the medullar area the collector tubes are reduced in numbers, uniformly dilated, with a flattened epithelium, surrounded by immature connective tissue without a tendency towards maturation into inflammatory cell infiltrated collagen (lymphocytes, plasma cells). Although many times it may have a normal aspect, the nephron can be functionally incompetent (Schulze and col., 1998).

Generally, hypoplasia is associated with compensatory hypertrophy of the congener kidney.

Renal dysplasia is a structural organization disorder that follows an abnormal differentiation during the period of nephrogenesis. The lesion may be uni- or bilateral, generalized or focalized. Histologically, dysplasia is highlighted through:

- non-synchronous differentiation of the nephrons, not corresponding to age;
- the persistency of primitive mesenchyme represented by a myxomatous type connective tissue;
- the persistency of metanephritic ducts;
- adenomatoid looking atypical tubular epithelium;
- the presence of osseous or cartilaginous tissue islands.

Renal dysplasia is a familial nephropathy seen in the following breeds: Chow-Chow, Samoyed, Doberman, Pincher, Norwegian Elkhound, Bull Terrier, Cocker Spaniel, Lhasa Apsos, Shih Tzu and Standard Poodle. In the youth of these breeds, the nephropathy with an autosomal dominant, autosomal recessive or sex-linked transmission represents the major cause for chronic renal insufficiency (Jansen and col., 1986; Brown and col., 1990; Picut and Lewis, 1987; Darrigrand and col., 2006).

Secondary to the renal dysplasia we can see interstitial fibrosis, renal cysts, glomerular hypercellularity (compensatory phenomena) (Zachary and McGavin, 2012).

The welding of the kidneys occurs during the nephrogenesis period being accomplished at one of the two poles (cranial or caudal) through a bridge formed by parenchyma that may or may not embody the bassinets, the ureters remaining separate. Thus, through fusion, we will see the formation of a structure with the appearance of a single, large kidney (horseshoe kidney), with two ureters, and with a normal histological structure and function (Zachary and McGavin, 2012).

Atrophy represents the reduction in the volume of the kidney that has reached its full morphofunctional maturity, following the diminishing of trophicity. It is secondary to vascular lesions, hydronephrosis, compression exercised by large tumors, retention or parasitic cysts. The atrophy of the epithelium of the nephrons is accompanied by the thickening of the basal membranes and is characteristic for chronic tubular lesions.

Macroscopically, the kidney is slightly smaller and with increased consistency.

Histologically, tubular atrophy was observed gradually, beginning with the shrinking of the tubular lumen from the medullar area following the direction of the compression force, the flattening and progressive atrophy of the epithelium down to its disappearance, the curling and considerable thickening of the tubular basal membranes. Finally, the uriniferous tubules will be replaced with connective tissue.

Agenesis represents the uni- or bi-lateral absence of the kidneys. Sometimes the kidney may be represented by only an embryonic vascular-connective bundle.

Aplasia, it is rare, and it represents the partial development of the kidney. Unilateral aplasia affects mostly the left kidney; it evolves associated or not with the hypoplasia or absence of the ureter, the hypoplasia of the opposite kidney or even other anomalies.

Hypertrophy represents the development of the kidney above the normal limits. It may be **compensatory**, in which case it is installed following the agenesis, aplasia, hypoplasia or surgical ablation of the opposite kidney. The healthy kidney does not undergo hypertrophy through nephron neoformation, but **cellular over-dimension** of the existing ones. In the case of total functional impairment of a kidney, the congener organ increases its size up to the normal wight of both kidneys in young animals, and up to 70% of their weight in adult ones (Coman and col., 1996).

Cytomegaly or renal megalocytosis is characterized through nuclear-cytoplasmic gigantism of the nephrocytes and is located inside the proximal contort tubules. It is caused by aflatoxin, nitrosamine and pyrrolizidine alkaloids from plants belonging to *Senecio, Cynoglossum, Echium, Crolalaria* genera, etc. (Coţofan O., 1992; Pop I., 2004).

Hydronephrosis, congenital or acquired, represents the dilation of the internal space of the kidney associated with the progressive compression atrophy of the renal parenchyma. Ureteral obstructions cause uni-lateral lesions, those affecting the urethra, bi-lateral lesions. In dogs, the most frequent causes for hydronephrosis are the decrease of the urinary transit following congenital malformations, urinary calculi, chronic urocystitis, urinary bladder or prostate neoplasia (Zachary and McGavin, 2012)**.** The glomerular filtration that continues even when there is an obstacle present in the extrarenal urinary tract will lead to the progressive accumulation of urine in the distal segments of the nephron, the atrophy of the papillae, of the pyramids and later of the cortex and the replacement of the normal morphofunctional elements with neoformed connective tissue. Finally, the kidney will appear as an immense, irregular sack, delimited at the periphery by a thin remanence of atrophied renal parenchyma

Osseous metaplasia can be seen in the hydronephrotic kidney, the transition cell epithelium simulating the transformation of mesenchymal cells into osteoblasts (Jubb and col., 2007).

Renal cysts are cavitary formations present in the renal parenchyma.

The shape and volume of the kidneys are dependent by the number and size of the cysts, which may be **congenital** or **acquired**. We hence feel the need to mention several aspects:

- the single renal cyst - affects a lobe. The volume of the cysts, although variable, may be greater than the one of the remaining kidney. The wall is thin, smooth, fibrous, lined on the inside with a shinny epithelium, the cavity containing a watery, colourless, odourless liquid, without urine smell.

- the polycystic kidney is usually bilateral. The cortical area is deformed by numerous cysts, creating the resemblance to a grape. We will find multiple cavities, of variable sizes, with a diameter of about 2-6 mm, separated by fibrous tissue of debris of atrophied parenchyma. When the cysts are numerous, the kidneys are very enlarged, pale and with a spongy aspect. This disorder appears to result from an anastomosis defect between the secretory and the excretory segments of the tubes, with the accumulation of urine and cystic distension of the proximal parts of the nephron. Congenital renal cysts can develop along with cysts inside other organs as well.

Histologically, we will observe the wall of the cysts, formed of densified collagen fibres and lined with a flattened epithelium, and around the cyst, the renal parenchyma will be compressed and fibrotic.

Polycystic kidney disease (PKD), being a **dominant autosomal hereditary disease** has been observed in dogs belonging to Cairn Terrier, West Highland White Terrier and Collie breeds, up to 6 months of age, as well as in Persian cats. Along with this lesion bi-lateral cysts were also observed. In the majority of cases, the abdomen was enlarged following the enlargement of the liver and kidneys (Gough and Thomas, 2004; Sellers and Richie, 1978).

The causes of this disease appear to be the dysfunctions of proteins *polycystin-1* and *polycystin-2* as a result of mutations in genes PKD-1 or PKD-2, involved in the synthesis of these proteins. The formation of tubular cysts is owed to mutations located on both alleles of these genes, PKD-1 and PKD-2 (Carone and col., 1994, Angus K.W., 1990).

Polycystin-1 is involved in the normal proliferation of cells and the mechanism of apoptosis. Also, this protein has a very important role in cellular adhesion, being a main component of desmosomes. Its absence or the formation of a mutant protein would lead to a faulty differentiation of tubular epithelial cells with the loss of polarity and an abnormal arrangement of cells within the structure of the uriniferous tubules, decreasing tubular absorption and determining the distension of the uriniferous tubules with the formation of tubular cysts (Schultze and col., 1998).

Polycystin-2 is located inside the Ca^{2+} canals of the cytoplasmic membranes.

In Bull Terriers, besides the polycystic kidney, nodular thickening of the mitral and aortic valves could be observed. Patients with ages between 6–15 months presented with hematuria and severe cardiac insufficiency (Gough and Thomas, 2004).

Acquired renal cysts are generally small and numerous. The aetiology also includes hypokalemia. The differential diagnosis should include hydatidosis. In dogs, the lesion may be bi-lateral.

Histologically, we may observe both glomeruli and uriniferous tubules distended and filled with a homogenous, clear material (sometimes basophilic), which, through progressive accumulation, leads to the atrophy and disappearance of the glomerular vascular bundle and the tubular epithelium. We may also observe the thickening of the basal membranes of both the glomerular capsule and the uriniferous tubules through the formation of connective tissue with laminar aspect and concentric arrangement. Glomerular and tubular cysts may also develop following extensive cortico-medullary fibrosis.

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MALIGNANCY – ASPECTS TO CONSIDER IN HISTOPATHOLOGICAL DIAGNOSIS

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Abstract
The histopathological exam is the most important tool when formulating a diagnosis in tumoral diseases. When we consider the examination of a tumor, the prognosis of the disease often depends of the character of that neoplasia (benign or malignant). To properly evaluate the future behavior of a neoplasia means to carefully observe and characterize several specific features. An abnormal differentiation degree of the cells can sometimes determine the presence of abnormal, monstruous cells, with little homogeneity between tumoral cells and a high rate of anisocytosis, anisokaryosis, anisonucleoliosis, multiple nuclei and various types of indentations or expansions of the structures of the cells. The mitotic index is another factor to take into consideration. A high mitotic index will always indicate a malignancy, and if it is associated with abnormal mitotic figures, the severity of the disease is even worse. Along with a multitude of other factors that need to be evaluated when establishing a diagnosis, from a histopathological point of view, the two mentioned criteria are the ones that need the most attention.
Key words: *tumor, differentiation degree, mitotic index*

Introduction

A tumor is a newly formed tissular mass (Coțofan, 1992). The influence that it has both locally, on the surrounding tissues and organs, as well as generally, on the entire organism, depends mostly on its benign or malignant character. This characteristic can only be determined based on a histopathological examination (Meuten, 2017).

The growth pattern of the tumor can be a first criteria for differentiation. Malignant tumors tend to be more infiltrative, whilst benign ones are better delimited, either through an actual fibrous capsule, or a pseudo-capsule created by the compressed surrounding tissue (Grant, 2016).

The invasion of the circulatory or lymphatic system is another characteristic of malignant cells and this derives from their lack of cohesion and also form their ability to synthesize substances that will create breaches in the blood vessels or lymphatic vessels walls. The result of any type of tumoral dissemination is usually metastasis (Morris, 2001; North, 2009).

Affecting local, regional or distal lymphnodes is another consequence of lymphatic dissemination and interferes severely with the intensity of the immune response (Kumar, 2015).

To establish a correct diagnosis and evaluate the future behavior of a tumor we need to evaluate the differentiation degree of the tumoral cells and the mitotic index. The first criteria shows us how much the mutations have transformed the initial cells so that they not resemble the cells of origin anymore, but also sometimes they do not resemble one another (Klopfeisch, 2016; Meuten, 2017).

The second criteria helps us understand how much that tumor will grow in the nearest future and if the mitosis that already exist will result in normal or abnormal cells (Klopfeisch, 2016; Meuten, 2017).

Materials and Methods

We examined several tumors received at the Laboratory of Anatomic pathology of the Faculty of Veterinary Medicine of Iași. The tumors were fixated in 10% formaldehyde solution, processed using the paraffin inclusion method, sectioned at 5 µm and stained using Masson

trichromic method. The microscopical examination was done using a Leica DM750 optical microscope with included camera.

Results and Discussions

It is clear that as tumoral cells become less differentiated, their ability to behave in an abnormal manner increases. Tumoral cells of epithelial origin may proliferate following more or less the pattern that the original cells would have (Withrow, 2013). For example, this cholangiocarcinoma still has a tendency to respect the arrangement of cells into canals, but already we can see a deviation from that standard, as well as anisocytosis, anisokaryosis, multiple mitotic figures and a variable chromatin pattern (Fig. 1).

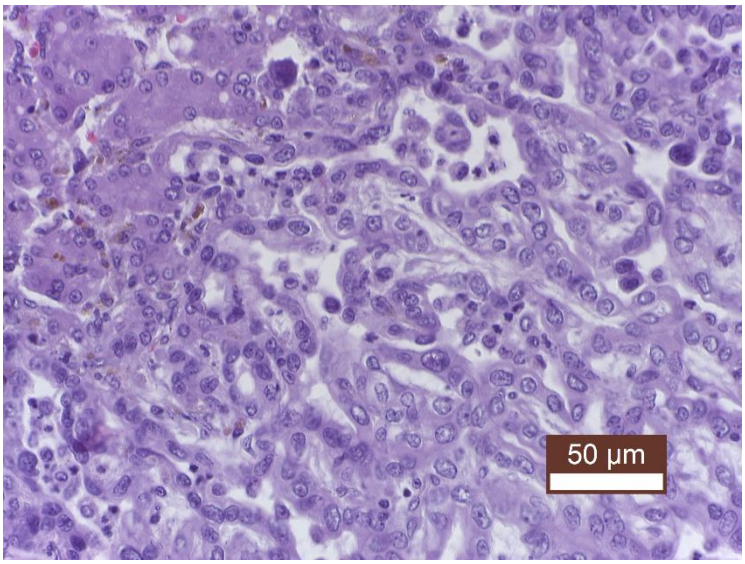

Fig. 1 Anaplasia, dysplasia and multiple mitotic figures. Cholangiocarcinoma. Dog. Masson trichrome stain

The variations between cells within the same tumor may also target the shape of the cells, as well as the size (Baba, 2002). In this image of a fibrosarcoma we can see that neoplastic fibroblasts have both round and spindle shapes (Fig. 2), along with other variations in cellular size, nuclear to cytoplasm ratio and nuclear shape.

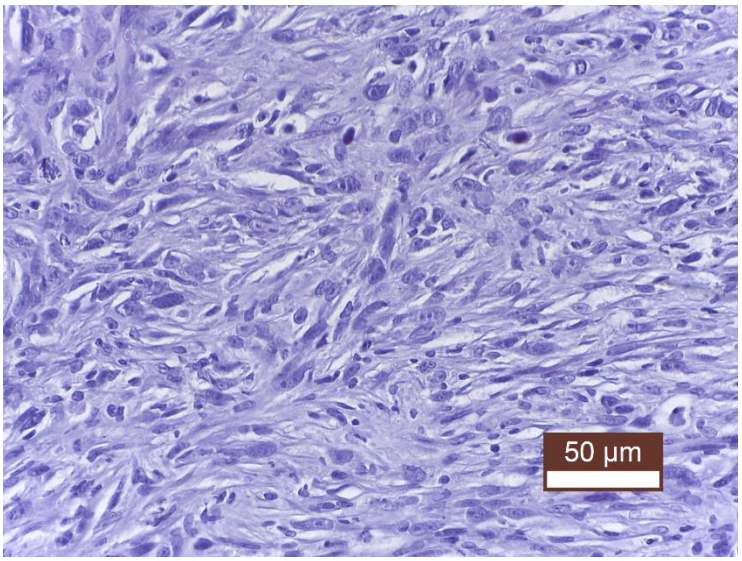

Fig. 2. Variations in cellular size and shape. Fibrosarcoma. Masson trichrome stain

The number, size and shape of the nucleoli can also vary between tumoral cells, along with anisokaryosis and inconsistant chromatin pattern (Fig. 3).

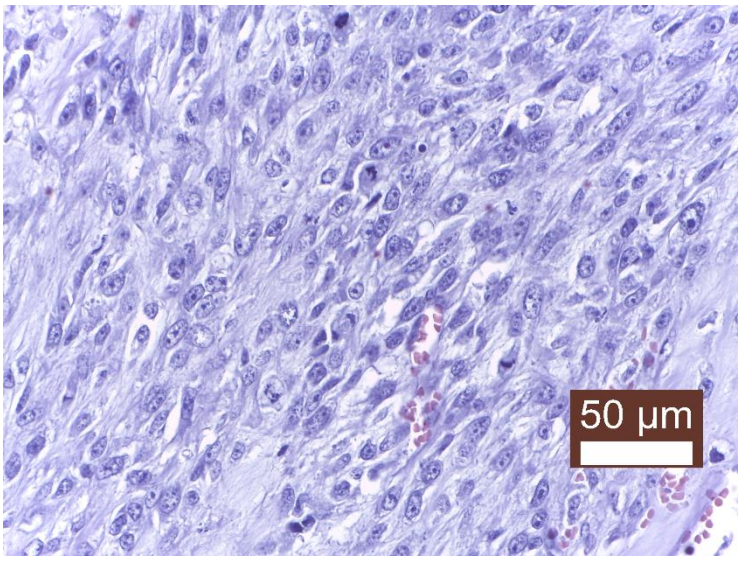

Fig. 3. Cells with variable number of nucleoli, of various shape and size. Fibrosarcoma. Dog. Masson trichrome stain

Another criterion that is almost specific for malignant tumors is the presence of metaplasia (Zachary, 2012; Meuten, 2017). Most of the times we will observe myxoid, cartilaginous or osseous metaplasia in tumors of mesenchymal origin, or corneous metaplasia in those of epithelial origin (Fig. 4, 5).

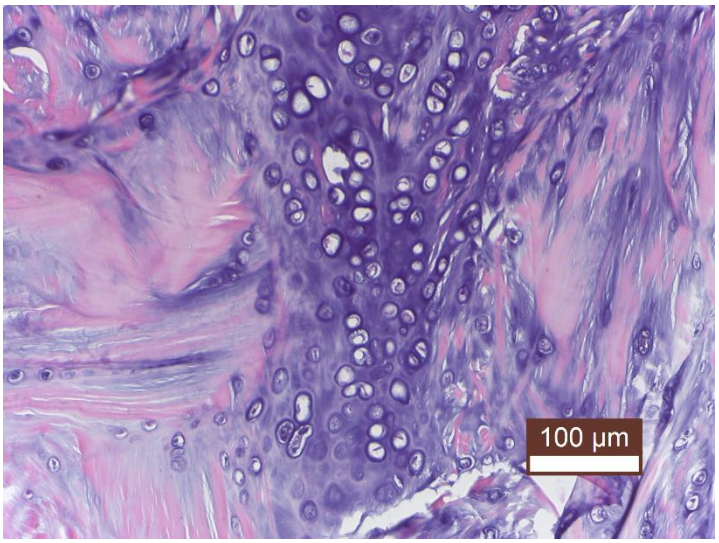

Fig. 4. Fibrosarcoma. Cartilaginous metaplasia. Dog. Masson trichrome stain

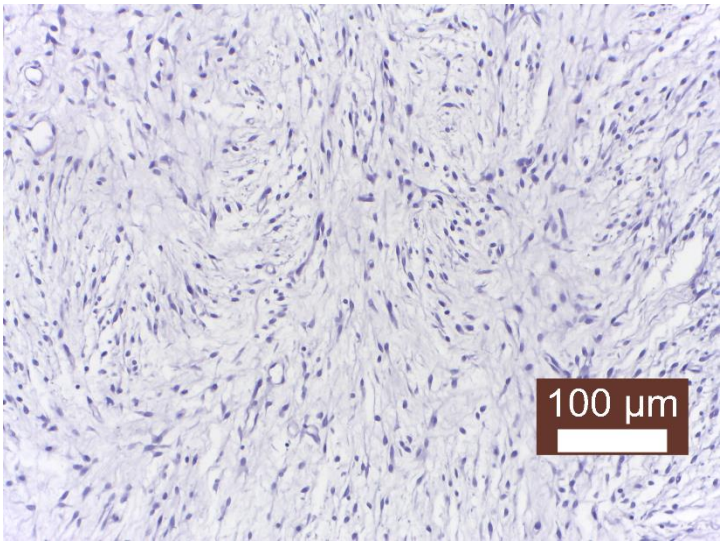

Fig. 5. Myxoid metaplasia. Sarcoma. Dog. Masson trichrome stain

Because of their rapid evolution, malignant tumors can sometimes fail to offer an optimal level of nutrients and oxygen to all their cells, which is why it is not uncommon to find within their structure necrotic areas (Fig. 6) or even mineralized necrotic debris (dystrophic mineralization – Fig. 7).

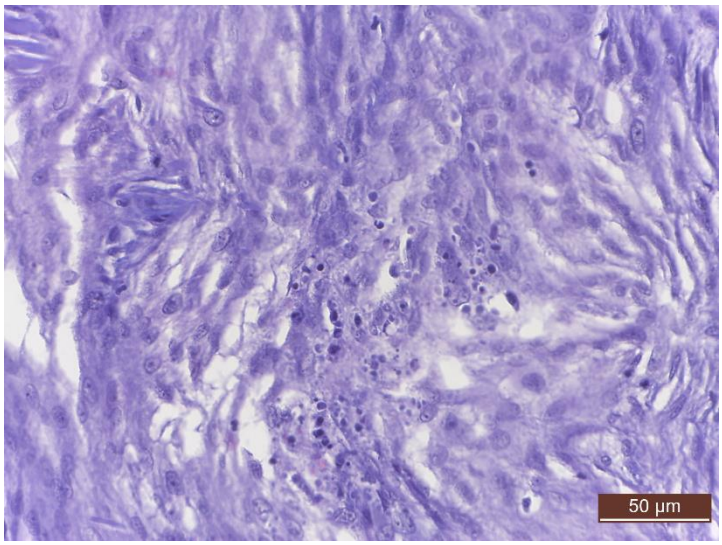

Fig. 6. Mineralization area inside a fibrosarcoma. Dog. Masson trichrome stain

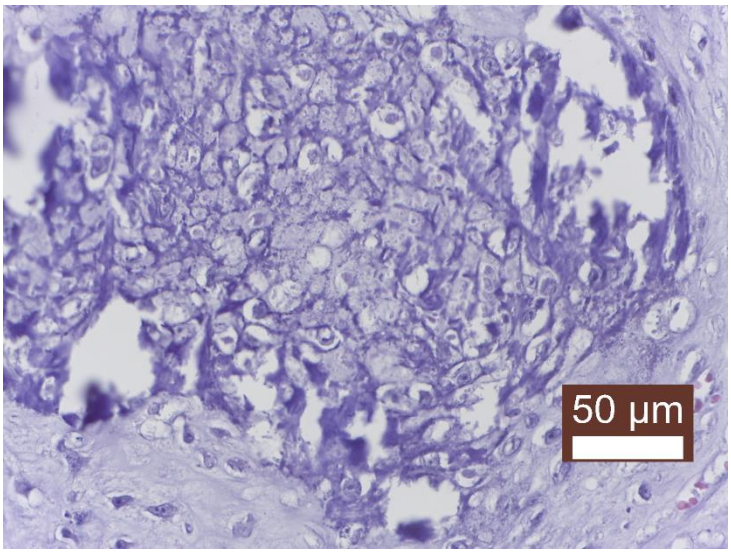

Fig. 7 Mineralization area. Fibrosarcoma. Masson trichrome stain

Conclusions

Although these are not the only criteria that need to be considered when establishing the malignancy of a tumor, the histopathological diagnosis should always evaluate the presence of anaplasia, dysplasia, mitotic figures or metaplasia.

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A SIMPLE BRAIN ATLAS OF THE ADULT ZEBRAFISH (DANIO RERIO)

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Abstract
The zebrafish and its brain popularity in scientific research created the necessity of a quick and simple atlas for the examination of the entire central nervous system structure. Sagittal sections of 5 µm were obtained from 10 adult both male and female zebrafish. Luxol Fast Blue-Cresyl Violet staining was performed in serial brain sections from whole adult zebrafish brain for examination of myelin and neuron structure and localization. Neurons can be well established with distinct cell bodies and nuclei in examined cerebral structures from both hemispheres. Cresyl violet is used to stain the neuronal cell bodies and processes which appear in a pink to violet color. Luxol fast blue stain is used to identify myelin in nervous tissue which gets stained in bright blue. The serial brain sections staining with Luxol fast blue-Cresyl Violet gives a quick and complete view of the central nervous system morphology and could be a useful tool in toxicology studies or in the research of neurological and neurodegenerative diseases.
Key words*: zebrafish, brain, atlas, Luxol Fast Blue-Cresyl Violet*

Introduction

The zebrafish model has proven its advantages for research not only by his shorter body size and lifespan compared to other vertebrate models, but most important by his genomic resemblance to human genome. Thus, many genes involved in human neurological disorders have very well-preserved orthologues in the zebrafish genome, suggesting that the biochemical events underlying the pathogenic mechanisms could be validated in the zebrafish nervous system. In addition, zebrafish show the organization of the central nervous system (CNS) like other vertebrates. Many neural circuits and cell types relevant to the study of human disorders and diseases are preserved in the brain of the zebrafish (Burton, E.A., 2015; Bradford Y. et al., 2017). Also, close correlations have recently been demonstrated between the levels of neurotrophins in various segments of the nervous system and the occurrence of neurodegenerative or neurological diseases in humans (Panula P. et al., 2010; Fontana et al., 2018).

The evolution of research on the CNS in zebrafish has created the need for a more detailed and explicit atlas of the brain of this fish. It was desired to realise a map of the main topographic regions of the brain based on which to easily identify the physiological location of various molecules. This atlas aims to highlight in color the neural populations and nerve pathways in the brain of the adult zebrafish, the most important nuclei and nervous paths of the CNS to facilitate the work of screening for various morphological changes at cellular level, or transcripts of genes or proteins derived from them at molecular level.

Materials and methods

10 healthy adult zebrafish were euthanized by immersion in cold water (4° C). The fixation was performed with 4% paraformaldehyde. Serial cross sections through the fish brain were performed. Samples dehydration was performed by usual method with alcohol series and cleared with xylene. Paraffin cubes were prepared and cut in slices of 5 µm by microtome. Serial sections of the brain (approx. 100 slides/fish) were stained with Luxol Fast Blue-Cresyl Violet and examined in light microscopy. Cresyl violet stained the neuronal cell bodies and processes which appear in a pink to violet color. Luxol fast blue highlights myelin in nervous tissue which gets

stained in bright blue. All areas of the brain were photographed and most significant parts were illustrated. The processing of the images was performed using the Adobe Photoshop program.

Results and discussions

The telencephalon initiates with the paired protuberances called olfactory bulbs that receive the information from the olfactory mucosa thru the olfactory nerve (I) and reach the glomerular layer of the bulbs (fig.1).

The secondary olfactory structures are the lateral and medial olfactory tracts (LOT/MOT) that originate in the mitral cells and connect the olfactory bulbs with the rest of the telencephalon, having implications in sexual (MOT) (fig.2) and feeding behavior (LOT) (fig.3) (Hara T.J., 1992; Kasumyan A. O., 2004).

Each of the olfactory bulb is composed of 4 concentric layers: primary olfactory fiber layer (POF), the most peripheral and rostroventral; glomerular layer (GL); external cellular layer (ECL), containing the large mitral cells; and the internal cellular layer (ICL) (fig.1).

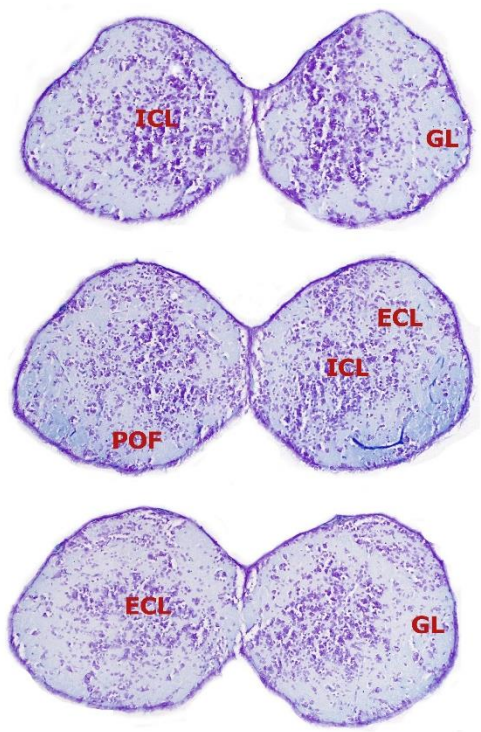

Fig. 1. Serial cross sections of the most rostral telencephalic regions-olfactory bulbs- in adult zebrafish. ECL- external cellular layer of olfactory bulb including mitral cells; ICl- internal cellular layer of olfactory bulb; GL- glomerular layer of olfactory bulb; POF- primary olfactory fiber layer; Luxol Fast Blue-Cresyl Violet X 200.

The main telencephalic body is divided into a dorsal (D) and a ventral (V) telencephalic area, each of them including a dorsal (Dd/Vd), central (Dc/Vc), lateral (Dl/Vl), medial (Dm/Vm) and posterior (Dp/Vp) nucleus (for V) or cell mass (for D) (fig. 3).

Ventral to Dp is located the nucleus taeniae (NT) that, together with Dp, receives part of the secondary olfactory projections. Beside the Vd, other three nuclei line the periventricular area of the V: ventral (Vv), supracommissural (Vs) and postcommissural (Vp) nuclei (fig. 3).

A group of nuclei from the V migrated from the ependymal area of the ventricle and are located mostly periferally. These nuclei are the central/commissural nucleus (Vc) and the lateral nucleus (Vl) (fig.3).

Diencephalon is divided into five main regions located dorsoventrally: epithalamus, dorsal thalamus, ventral thalamus, posterior tuberculum, hyphothalamus and an intermediate region between the telencephalon and diencephalon, the so called area preoptica. The last one includes dorsal and ventral nuclei, most rostral being the anterior parvocellular preoptic nucleus (PPa) continued by the posterior parvocellular preoptic nucleus (PPp) and the suprachiasmatic nucleus (SC), which are located ventrally (fig.4).

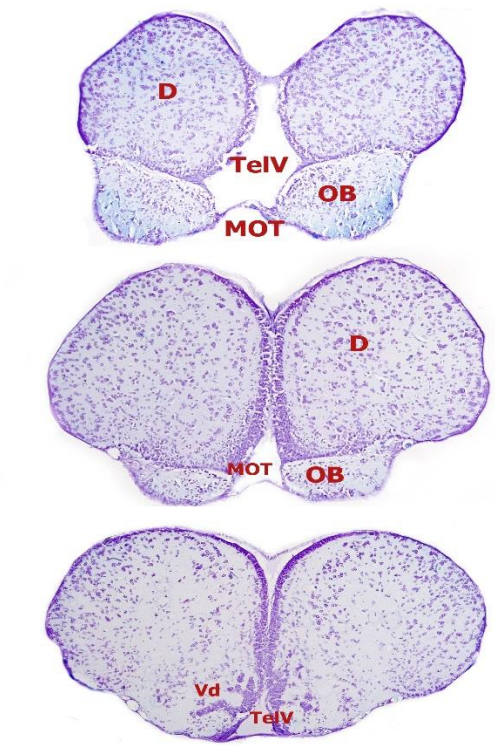

Fig. 2. Serial cross sections of rostral telencephalon in adult zebrafish. D- dorsal telencephalic area; MOT- medial olfactory tract; OB- olfactory bulb; TelV- telencephalic ventricles; Vd- dorsal nucleus of ventral telencephalic area; Luxol Fast Blue-Cresyl Violet X 200.

Dorsally are found the magnocellular preoptic nucleus (PM) and the gigantocellular part of magnocellular preoptic cells (PMg). The preoptic area also contains CRF-expressing neurons that are part of the stress axis and which project onto the anterior pituitary gland (Bally-Cuif, L., & Vernier, P., 2010). Epithalamus is formed by nuclei (dorsal (Had) and ventral (Hav) nuclei of habenula) and two protuberances. The most important structure is the epiphysis, also called the pineal gland, with main roles in the regulation of the circadian cycle and melatonin hormone synthesis.

The preoptic area and the thalamus are some of the main regions of the brain, together with optic tectum, pretectum and accessory optic system, that receive information from the retina (fig. 5).

The largest and most ventrally diencephalic region, hypothalamus, consists of a dorsal, ventral and a caudal zone and also a hypothalamic ventricle, an extension of the diencephalic ventricle (DiV). The ventral and caudal zones form most of the tuberal hypothalamus (TH), while the dorsal region consists mainly of the paired inferior lobes of hypothalamus (IL), into which the DiV prolongs (fig.5; fig.6).

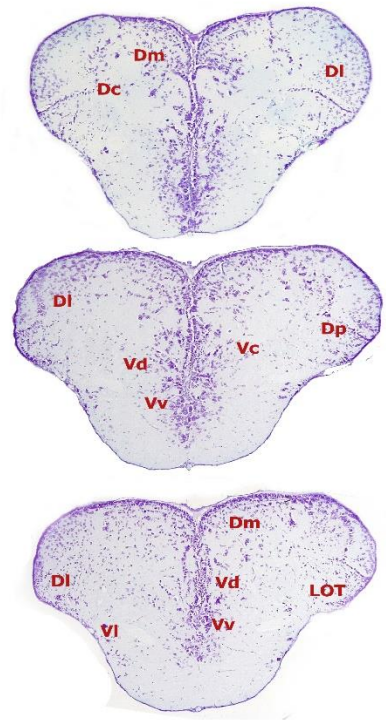

Fig. 3. Serial cross sections of caudal telencephalon in adult zebrafish. Dc- central zone of dorsal telencephalic area; Dm- medial zone of dorsal telencephalic area; Dl- lateral zone of dorsal telencephalic area; Dp- posterior zone of dorsal telencephalic area; LOT- lateral olfactory tract; Vc- central nucleus of ventral telencephalic area; Vd- dorsal nucleus of ventral telencephalic area; Vl- lateral nucleus of ventral telencephalic area; Vv- ventral nucleus of ventral telencephalic area; Luxol Fast Blue-Cresyl Violet X 200.

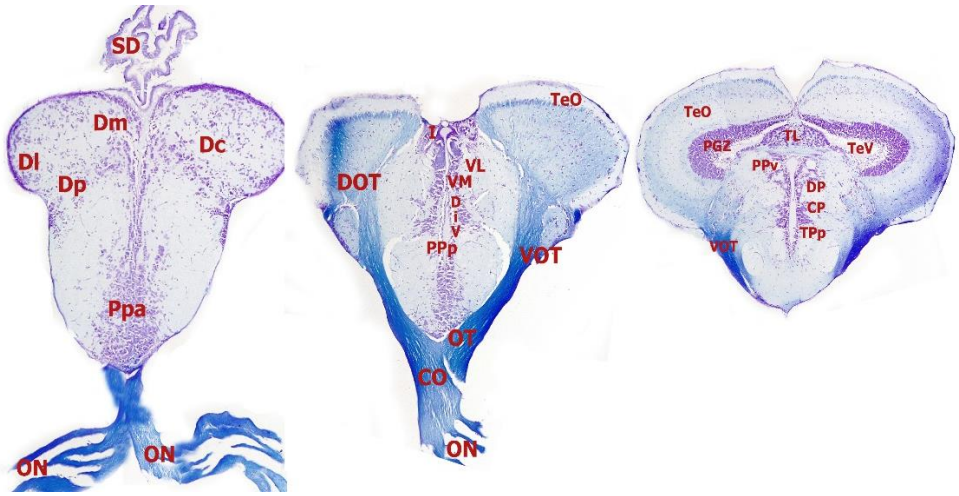

Fig. 4. Serial cross sections of caudal telencephalon, diencephalon and rostral mesencephalon in adult zebrafish. CP- central posterior thalamic nucleus; Dc- central zone of dorsal telencephalic area; DiV- diencephalic ventricle; Dl- lateral zone of dorsal telencephalic area; Dm- medial zone of dorsal telencephalic area; DOT- dorsomedial optic tract; Dp- posterior zone of dorsal telencephalic area; DP- dorsal posterior thalamic nucleus; ON- olfactory nerve; OT- olfactory tract; PGZ-periventricular grey zone of optic tectum; PPa- parvocellular preoptic nucleus, anterior part; PPp- parvocellular preoptic nucleus, posterior part; PPv- periventricular pretectal nucleus, ventral part ; SD- dorsal sac; TeO- tectum opticum; TeV- tectal ventricle; TL- torus longitudinalis; TPp- periventricular nucleus of posterior tuberculum; Val- lateral division of valvula cerebelli; VL- lateral thalamus; VOT- ventrolateral optic tract; VT- ventral thalamus. Luxol Fast Blue-Cresyl Violet X 400.

The hypothalamus projects to the area preoptica, hypophysis and telencephalon and play a role in feeding behavior, reproduction, food intake regulation and aggressive behaviors. Also it was found that it is implicated in the regulation of sleep, heart rate and blood pressure, and the control of body temperature (Bally-Cuif, L., & Vernier, P., 2010).

The mesencephalon has a very large and complex dorsal structure called optic tectum (TeO) and two more ventrally and reduced ones: torus semicircularis (TS) and tegmentum (fig. 5; fig.6). Torus semicircularis receives projections from the telencephalon and consists of a central (TSc) and a ventrolateral nucleus (TSvl), with role in hearing and mechanoreception (fig.5).

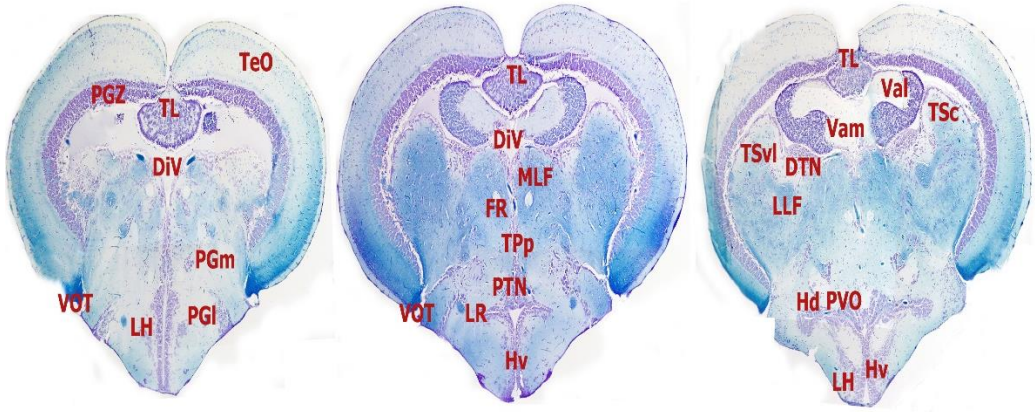

Fig. 5. Serial cross sections of mesencephalon and rostral metencephalon in adult zebrafish. DiV- diencephalic ventricle; DOT- dorsomedial optic tract; DTN- dorsal tegmental nucleus; Hd- dorsal zone of periventricular hypothalamus; FR- fasciculus retroflexus; Hv- ventral zone of periventricular hypothalamus; LH- lateral hypothalamic nucleus; LLF- lateral longitudinal fascicle; LR- lateral recess of diencephalic ventricle; MLF- medial longitudinal fascicle; PGl- lateral preglomerular nucleus; PGm- medial preglomerular nucleus; PGZ-periventricular grey zone of optic tectum; PTN- posterior tuberal nucleus; PVO- paraventricular organ; TeO- optic tectum; TeV- tectal ventricle; TL- torus longitudinalis; TPp- periventricular nucleus of posterior tuberculum; TSc- central nucleus of torus semicircularis; TSvl- ventrolateral nucleus of torus semicircularis; Val- lateral division of valvula cerebelli; Vam- medial division of valvula cerebelli ; VL- lateral thalamus; VOT- ventrolateral optic tract; VT- ventral thalamus. Luxol Fast Blue-Cresyl Violet X 400.

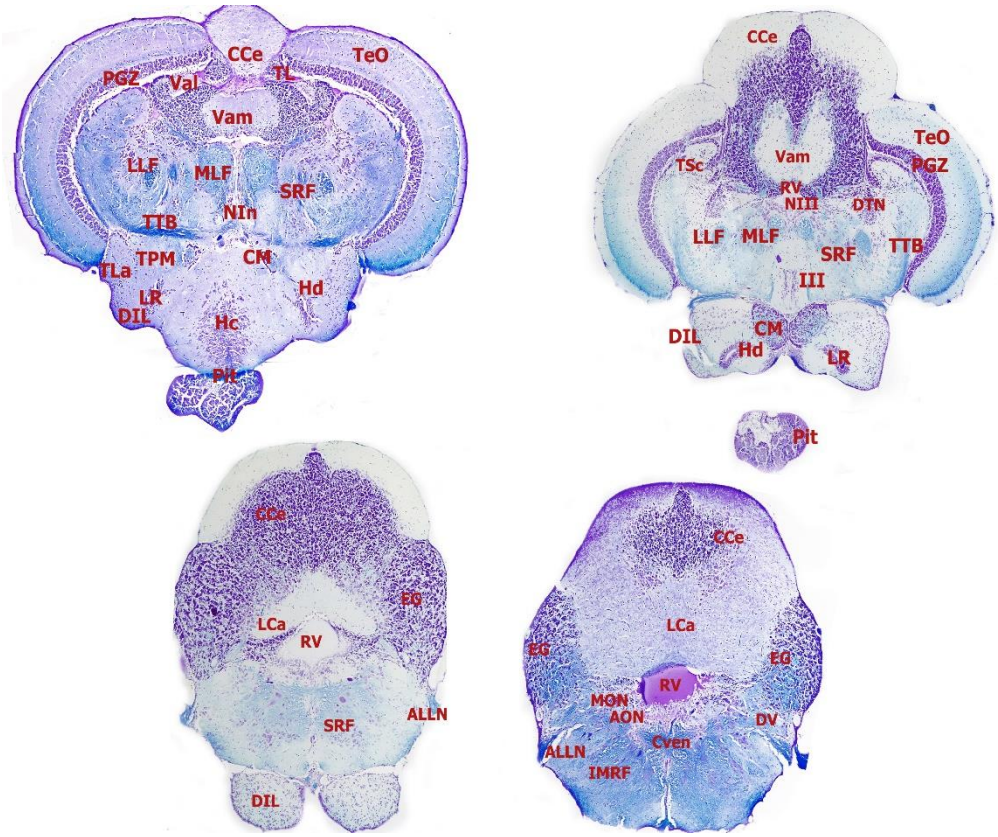

Fig. 6. Serial cross sections of mesencephalon and rhombencephalon in adult zebrafish. ALLN- anterior lateral line nerves; AON- anterior octaval nucleus; CCe- corpus cerebelli; CM- mammillary body; Cven- commissura ventralis rhombencephali; DIL- diffuse nucleus of the inferior lobe; DV- descending trigeminal root; Hc- central zone of periventricular hypothalamus; GC- griseum centrale; Hc- caudal zone of periventricular hypothalamus; Hd- ventral zone of periventricular hypothalamus; IMRF- intermediate reticular formation; LLF- lateral longitudinal fascicle; LCa- lobus caudalis cerebelliLR- lateral recess of diencephalic ventricle; MLF- medial longitudinal fascicle; MON- medial octavolateralis nucleus; NIII- oculomotor nucleus; Nln- nucleus interpeduncularis; ; NLV- nucleus lateralis valvulae; Pit- pituitary; PGZ-periventricular grey zone of optic tectum; RV- rhombencephalic ventricle; SRF- superior reticular formation; TeO- optic tectum; TL- torus longitudinalis; TLa- torus lateralis; TPM- pretecto-mammillary tract; TSc- central nucleus of torus semicircularis; TTB- tecto-bulbar tract; Val- lateral division of valvula cerebelli; Vam- medial division of valvula cerebelli; III- oculomotor nerve. Luxol Fast Blue-Cresyl Violet X 400.

The tegmentum is the most ventral mesencephalic structure and includes cranial nerve nuclei with major role in motor functions (oculomotor-NIII; trochlear-NIV) and parts of the reticular formation (fig.6). It continues with medulla oblongata without a clear demarcation (Wullimann M.F. et al., 1996).

Optic tectum, the most complex layered structure in the zebrafish brain, consisting of neuronal bodies and axons and receives visual information concerning the shape, movement and colour from the retina, thalamus and pretectum. It also integrates inputs from telencephalon, torus semicircularis and reticular formation.

The optic tectum contains three main areas: the superficial and central zones, where the tectal afferents end; the periventricular zone (SPV), where the majority of the tectal cell bodies reside. The outermost layers are stratum marginale (SM) and stratum opticum (SO) that form the superficial zone, followed by stratum fibrosum et griseum superficiale (SFGS), stratum griseum centrale (SGC) and stratum album centrale (SAC) forming the central zone.

The innermost layer, periventricular grey zone of optic tectum (PGZ), is predominantly composed of pyriform neurons, while in the stratum marginale are predominantly found unmyelinated axons. The other layers contain cells scattered among neuronal processes from different sources (Bally-Cuif, L., & Vernier, P., 2010).

The rhombencephalon in zebrafish comprises a rostral division, metencephalon, and a caudal division, myelencephalon, separated only arbitrarily. The main segments of the rhombencephalon are the cerebellum and medulla oblongata (MO).

Medulla oblongata associates with the majority of cranial nerves and their primary motor and sensory nuclei: the trochlear (IV), trigeminal (V), abducens (VI), facial (VII), octaval (VIII), glossopharyngeal (IX) and vagal (X) nerves, as well as the lateral line nerves which includes a component for mechanoreception and electroreception. It also comprises the reticular formation, the raphe nuclei and many ascending or descending fiber tracts (Bally-Cuif, L., & Vernier, P., 2010).

The cerebellum is large and composed of three parts in zebrafish: the vestibulolateralis lobe which includes the medial caudal lobe (LCa) and the paired lateral eminentiae granulares (EO); the corpus cerebelli (CCe), and the valvula cerebelli, which has a medial (Vam) and a lateral subdivision (Val) (Wullimann M.F. et al., 1996).

The cerebellum conserves the classic architecture comprised of a three-layered cortex: the innermost layer is the granular layer, densely packed with granule cells, along with interneurons; Purkinje layer is the middle one and contains the Purkinje cell bodies; the outermost is the molecular layer, which contains the flattened dendritic trees of Purkinje cells (fig.6; fig.7). The main function of the cerebellum is in learning and was found to participate in both spatial and emotional cognition in delay conditioning assays (Yoshida et al., 2004; Rodriguez et al., 2005).
The reticular formation of the rhombencephalic segment is divided into a midline, a medial and a lateral column. The medial column includes the superior, intermediate, and inferior nuclei of the reticular formation, also called superior, intermediate, and inferior reticular formation (SRF/IMRF/IRF) (fig.6; fig.7) (Wullimann M.F. et al., 1996).

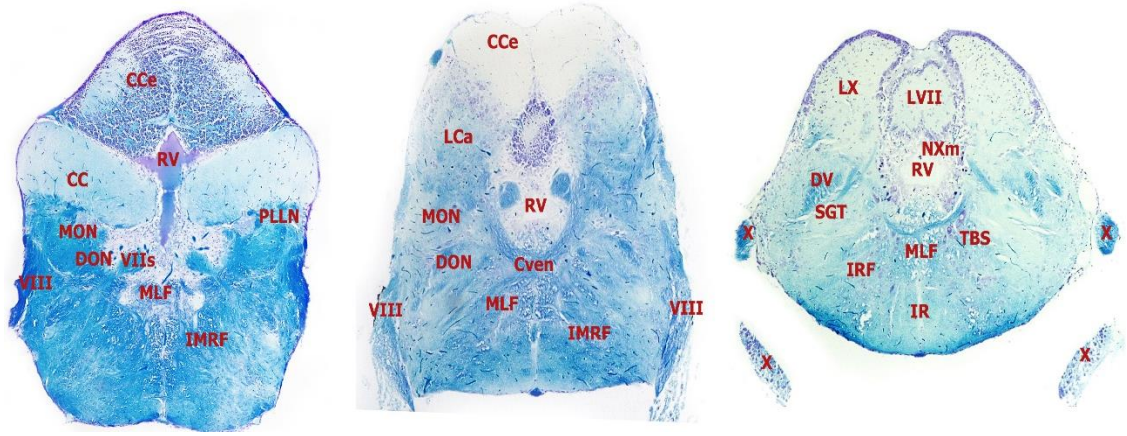

Fig. 7. Serial cross sections of rhombencephalon in adult zebrafish. CC- crista cerebellaris; CCe- corpus cerebelli; Cven- commissura ventralis rhombencephali; DON- descending octaval nucleus; DV- descending trigeminal root; IMRF- intermediate reticular formation; IR- inferior raphe; IRF- inferior reticular formation; LCa- lobus caudalis cerebelli; LVII- lobus facialis; LX- vagal lobe; MLF- medial longitudinal fascicle; MON- medial octavolateralis nucleus; NXm- vagal motor nucleus; PLLN- posterior lateral line nerve; RV- rhombencephalic ventricle; SGT- secondary gustatory tract; TBS- tractus bulbospinalis; III- oculomotor nerve; VIIs- sensory root of the facial nerve; VIII- octaval nerve. Luxol Fast Blue-Cresyl Violet X 400.

The most caudal region of the central nervous system, medulla spinalis or spinal cord, is constituted of the dorsal horn (DH) and of the dorsal root ganglia which extends peripheral axons under the skin to detect a range of mechanical, thermal and chemical stimuli and also of a ventral horn (VH) (fig.8).

The descending spinal projections originate in all divisions of the reticular formation, in the inferior raphe region (but not in the superior raphe), in vestibular and sensory trigeminal nuclei (fig. 7). Furthermore, the nucleus of the medial longitudinal fascicle is an ancestral craniate premotor system descending to medullary and spinal levels (fig. 7; fig.8) (Bally-Cuif, L., & Vernier, P., 2010).

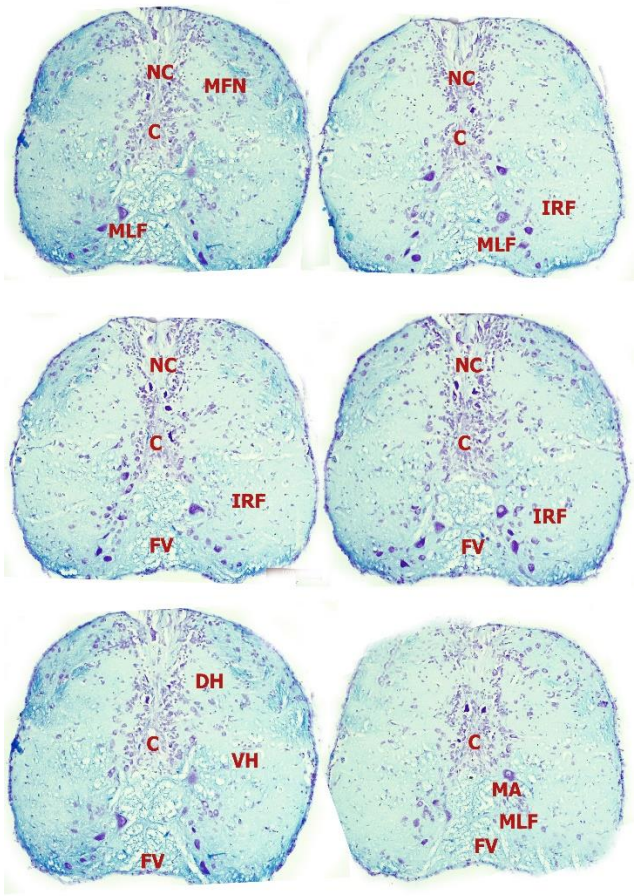

Fig. 8. Serial cross sections of medulla spinalis in adult zebrafish. C- central canal; DH- dorsal horn; FV- funiculus ventralis; IRF- inferior reticular formation; MA- Mauthner axon; MFN- medial funicular nucleus; MLF- medial longitudinal fascicle; NC- commissural nucleus of Cajal; VH- ventral horn. Luxol Fast Blue-Cresyl Violet X 400

Conclusions

Zebrafish shows the basic organization of the nervous system like other vertebrates. Many neural circuits and cell types relevant to the study of human disease are preserved in the central nervous system of this fish (Stewart A. et al., 2014).

The complexity of the anatomical and histological structure of the zebrafish brain is requiring complex research and understanding for the proper and useful manipulation of this fish as a reference model organism for neurogenesis, neuroplasticity, but also neurodegenerative disorders or toxicological mechanisms in other vertebrates, most inquired, in human.

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MONITORING LAMENESS IN A DAIRY COWS FARM

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Abstract
Lameness has an important impact on milk production, welfare and farm profitability. Monitoring cattle was done in a farm A with 1000 Holstein from Bacău County, in the east of Romania. The indices used to evaluate the herd lameness were: incidence rate of lameness for treated cases, recurrence rate of lameness for treated cases, lameness index, monthly incidence rate and monthly recovery rate of lameness. Data collected from each lame cow included its unique identification, a locomotion (mobility) score, the identity of the lame leg(s) and any other pertinent information. We were focused on four most common lesions associated with lameness: sole ulcers, white line disease, digital dermatitis and interdigital necrobacillosis. In the farm A, percentage of lame cow with score 2 was 25, and the percentage of severely lame (score 3) was 5, much higher comparing to the targets. All other lameness indices were much higher comparing to the targets of a normal farm, which may explain the fertility problems and a decreased milk production during the last 2 years. Once lame cows have been identified, they should be examined, diagnosed and treated as soon as possible. A combination of early detection and effective treatment may have a number of benefits to the cow, herd and farm.
Key word*: lameness, locomotion score, foot-trimming, dairy cows*

Introduction

Lameness is a disease in all dairy farms where animals are raised for high milk production whether they are indoors, at pasture or a combination of both systems. In UK dairy cattle, during winter, the mean herd prevalence was 23.9% and 21.1% in summer (Cook, 2003). Other studies in UK based on data from 227 dairy herds, the prevalence of lameness during winter was estimated at 36.8% (Barker et al., 2010). The financial losses associated with lameness come from infertility, slaughter reduction in milk yield and high treatment costs. In a study, the average cost of lameness in UK herd was 80 euro per cow (Willshire and Bell, 2009). In this study we focused on the most common lesions associated with lameness and indices used to evaluate the herd lameness.

Materials and methods

Monitoring cattle was done in a farm A with 1000 Holstein from Bacău County, in the east of Romania. Data collected from each lame cow included its unique identification, a locomotion (mobility) score, the identity of the lame leg(s) and any other pertinent information. The indices used to evaluate the herd lameness were: incidence rate of lameness for treated cases, recurrence rate of lameness for treated cases, lameness index, monthly incidence rate and monthly recovery rate of lameness. We were focused on four most common lesions associated with lameness: sole ulcers, white line disease, digital dermatitis and interdigital necrobacillosis.

Results and discussions

In this farm A lameness has been monitored and recorded poorly in comparation with mastitis and fertility. This may explain in part explain why lameness control has lagged behind that of other diseases. Usually, the treatment records can be used to estimate the incidence and causes of lameness and the success of treatment.

There are many risk factors for lameness. Wet, slurry contaminated surfaces are major risk factors for digital dermatitis and interdigital necrobacillosis, but also predispose cows to heel erosion, soft claw horn, sole bruising, sole ulcers and possibly white line lesions. Forcing cows to stand for more than 2 h/ day while waiting for milking, artificial insemination, pregnancy diagnosis or other routine management activities can predispose to foot lesions. Poor lying comfort can also

lead to prolonged standing. In the farm A, cows are on concrete surfaces with insufficient straw bedding, which may explain the high lameness indices.

The role of nutritional management and an adequate ration (carbohydrates, protein, trace minerals, and vitamins) are very important in the control of lameness. Cows with a low body condition score at calving and in early lactation are predisposed to suffer from lameness because condition score is positively correlated with the thickness of the digital cushion, and the prevalence of sole ulcers and white line disease are associated with the thickness of the digital cushion (Bicalho et al., 2009; Hoedemaker et al., 2009). The supplementation of diets with biotin (20 mg/cow/ day) leads to a significant reduction in the incidence of white line disease (Pötzsch et al., 2003). In this farm A, cows are not receiving any kind of vitamins and minerals supplements. Supplementing diets with methionine, zinc, manganese, copper and cobalt at different levels of inclusion and in different forms may improve claw health and reduce the incidence of claw horn lesions.

The four most common lesions associated with lameness found in the farm A were: sole ulcers (pododermatitis circumscripta), white line disease (white line separation or white line haemorrhage), digital dermatitis (Mortellaro disease) and interdigital necrobacillosis (foot rot, foul or interdigital phlegmon) (Figs. 1, 2).

Sole ulcer is characterized by exposed corium below the flexor process of the pedal bone. In white line disease, the junction between the sole and wall is affected, including bruising (haemorrhage), separation (fissuring) and the formation of abscesses. Digital dermatitis is a well-circumscribed infection of the skin, between the heel bulbs or palmar/plantar pastern area. Lesions start as exudative epithelial erosions/ulceration, progressing to granulation, followed by hyperkeratosis and scab formation. Interdigital necrobacillosis is an acute bacterial infection of the subcutaneous tissues of the interdigital space characterized by symmetrical swelling, separation of the claws and interdigital skin necrosis, with a pungent odor (Huxley et al. 2012). Sole haemorrhage and ulcers are considered the result of contusions and damage to the tissues lying under the distal phalanx. White line haemorrhage and separation may have a similar cause.

Digital dermatitis is caused by bacteria, a combination of *Treponema* spp. is implicated. The bacteria are in lesions on diseased feet, and the transmission is cow to-cow via the environment. Interdigital necrobacillosis is caused by *Fusobacterium necrophorum*, which is present in cattle faeces and shed into the environment.

Claw horn lesions lead to greater losses than other lesions, with sole ulcer resulting in the largest losses. Animals have low productions for some time after treatment. Lame cows produce less milk before they are visibly lame (Reader et al., 2011). Total milk lost varies by lesion, for example, in case of sole ulcer, approximately 600 kg milk per lactation is lost (Huxley et al. 2012). In the farm A there are a decreased milk production per cow in the last 2 years.

Fig. 1 Sole ulcer in a Holstein cow

Fig. 2 Heel horn erosion in a Holstein cow

Regarding the fertility, lame cows have reduced cyclicity because of anoestrus or cystic ovarian disease, have a lower conception rate (Melendez et al., 2003), an increased risk of conception failure (Hernandez et al., 2005) and require more services per pregnancy. Lame cows have long calving to first service intervals calving to conception intervals, numbers of days open and long calving intervals (Hultgren et al., 2004). The farm A have all this fertility problems during the last 2 years

Locomotion score data was used to estimate the prevalence of lameness, to identify animals for treatment and to measure the success of control programs over time. Many locomotion scoring systems have been described, but, the two most commonly used are those of Sprecher et al. (1997), who proposed a five-point scale from not lame through to barely able to stand, and Whay et al. (2003) and Archer et al. (2010), who proposed a four-point scale.

For routine on-farm monitoring it is recommending the four-point scale for its simplicity and versatility. This score provides: Score 0 (cow walks with even weight bearing and rhythm on all four feet, with a flat back, long, fluid strides are possible; Score 1 (cow steps are uneven with rhythm or weight bearing or strides shortened, affected limb or limbs are not immediately identifiable); Score 2 (cow presents uneven weight bearing on a limb that is immediately identifiable and/or obviously shortened strides, usually with an arch to the center of the back); Score 3 (cow is unable to walk as fast as a brisk human pace (cannot keep up with the healthy herd) and signs of score 2 (Archer et al., 2010).

In the farm A, percentage of lame cow with score 2 was 25, and the percentage of severely lame (score 3) was 5, much higher comparing to the targets of a normal farm. Key indices found in the farm A and targets recommended for lameness are presented in Table 1. Regular, consistent locomotion scoring of all the cows in the herd allows the farmer to create an action list of lame cows for treatment and to conduct important analyses.

The estimation of incidence rates of lameness at different times and in different groups of cows is very important. Incidence rate is the number of new cases of lameness in a group of

individuals at risk over a specified time period, and is usually expressed as cases per 100 cows per year. The incidence rate of lameness should be evaluated for different stages of lactation, for cows of different parity, in different groups of cows within the herd, at different times of the year and for different lesion types and severities. It can be assessed over a two-weekly, monthly, or three-monthly period (Huxley et al. 2012). In the farm A this index was 30, much higher comparing to the target (<10-20).

The recurrence rate of lameness for treated cases (percentage of cows treated for lameness in which the lameness recurs in a six-month period), is high, indicating that the treatment is not appropriate every time. All other lameness indices were much higher comparing to the targets of a normal farm.

Table 1.

Lameness indices in the farm A

Index	**Target** (Huxley et al. 2012)	**Farm A**
Incidence rate of lameness for treated cases (cases/100 cows at risk/year)	<10-20	30
Recurrence rate of lameness for treated cases (percentage of cows treated for lameness in which the lameness recurs in a six-month period)	<25	30
Lameness index (score2)	<10-15	25
Lameness index (score3)	<1-2	5
Monthly incidence rate of lameness defined by mobility score (cows moving into a lame category as a percentage of those in the non-lame category at the previous recording)	<1-5	10
Monthly recovery rate of lameness defined by mobility score (cows moving into a non-lame category as a percentage of those in the lame category at the previous recording)	>75	55

Early identification and effective treatment reduce the prevalence of lameness by shortening the length of time. This also leads to a reduction in the incidence of future lameness by reducing the number of repeat cases. The severely lame cows (score 3) must be treated as soon as they are identified and lame cows (score 2) within 2 days. The most successful farms treat all lame cows as they are identified. The early stages of foot disease are easier to treat, respond more quickly and fully to treatment and are less likely to recur (Huxley et al. 2012).

In the farm A is no regular program of early detection of lameness. Many cows (25%) remain for a long time with the claw overgrowth, foot imbalance (mediolateral claw imbalance) and lameness. Foot-trimming interval is larger than 1 year in this farm (Fig.3)

Foot-trimming intervals should vary according to need. All cows that are lame or have overgrown claws (>9 cm of the dorsal wall for Holsteins) should be examined and trimmed as soon as practically possible. Given that most claw lesions develop in the weeks following calving, preventive trimming immediately before or soon after drying off is an appropriate time for many herds. In housed animals, a further examination may be warranted in mid-lactation (Hernandez et al., 2007).

Foot bathing is one of the principal measures for controlling lameness associated with digital dermatitis, both as a primary disease and when it is secondary to other lesions (Evans et al., 2011). Numerous chemicals with disinfectant qualities are used as foot bath solutions for dairy cattle, including formalin (5%, range 2–10%), copper sulfate (5%, range 2–6%), peracetic acid (1–2%) and hypochlorite (2%). Formalin and copper sulfate are the most widely used agents, but are not recommended for animals with painful, ulcerated lesions.

In the farm A, it is used copper sulfate solutions (5%) and formalin (5%) as feet antiseptics (Fig. 4). Antibiotics (tetracyclines, lincomycin) are also used to treat complicated cases of lameness. The fact that the cows are not often observed and the foot-trimming interval is larger than 1 year, the lameness persists with a high percentage in this farm. It is recommended foot bathing after 4–6 milkings each week (either consecutive milkings or consecutive days), which can then be adjusted up or down depending on the prevalence of lameness lesions.

Fig.3 Foot-trimming in a Holstein cow with an incipient digital dermatitis

Fig. 4 Foot asepsis with copper sulfate solution

Conclusions

All the lameness indices from farm A, were much higher comparing to the targets of a normal farm, which may explain the fertility problems and a decreased milk production during the last 2 years.

Once lame cows have been identified, they should be examined, diagnosed and treated as soon as possible.

A combination of early detection and effective treatment may have a number of benefits to the cow, herd and farm.

Milk production and lameness have a heritable component so, it is recommended to select for cows that produce more milk and are less likely to become lame.

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GOAT BREEDING AND HERDS HEALTH STATUS IN THE NORTHEAST AREA OF ROMANIA – A SHORT REVIEW

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Abstract
*The goat is a species that managed to attract attention to the breeders due to the fact that they are suitable for an efficient holding in small households, have a high lactogenic capacity, high prolificacy, precocity and high degree of adaptability and resistance, occupying areas where the natural resources and field conditions do not allow the growth of other species, which is why the goat herds in our country is constantly growing. Thus, the goat population in Romania increased from 1009794 animals in 2005 to 2057309 in December 2018. Our country occupying the third place in the hierarchy of Member States of the European Union, in 2019, following countries with tradition in the goat breeding, such as United Kingdom and Spain. The most numerous are the Carpathian native goats and Banat White, a crossbreed obtained from the Carpathian and Saanen breeds, followed by French Alpine and Saanen, imported lately to improve milk production. To date, in Romanian literature had been reported researches on the subject of diseases and health problems in goats, there are studies on paratuberculosis, contagious ecthyma, infectious respiratory disorders, gangrenous mastitis, contagious agalactia, bluetongue, caprine arthritis and encephalitis, and parasitic infections as coccidiosis, tapeworm (*Monezia, Cestoda*) and round worms (*Nematoda - Trichostrongylus sp.) *infestation, etc. Although Romania has a national program for urveillance and control program for goat health status, the problems in livestock goat diagnosed and published in recent years, are poor and changing, unsystematic and insufficient. The epidemiological situation in Romania has undergone significant changes in recent years, our country is the southeastern border of the European Union, which implies an increased risk for all major animal diseases and, of course, monitoring programs and greater control than in other Member States of the European Union. An effective animal health program is an essential part of a successful dairy goat management program.*
Keywords*: goat, health status, infectious, metabolic, parasitic*

Introduction

The aim of the present paper is to analyze the goat health status in Romania and to present the reported data on the subject of diagnosed diseases. In Romania goat herds are mainly traditionally raised, their productive potential depends on the geographical area, the type of maintenance and feeds, the owner's experience and level of knowledge on goat farming. The Carpathian and Banat White breeds accounted for 70% of the national livestock population, with the proportion now decreasing due to the import of specialized breeds, especially for milk production - Saanen, French Alpine, Anglo Nubian, Murcina Granadina, German Nobility and Toggenburg. The main goal of goat's breeding is milk production and meat production, especially kids. Goat farming is mainly organized into mini farms (10-50 goats), whose main product is milk, that is usually processed into cheese in family farms. Better use of underutilized pastures, the country holds five million hectares of pastures mostly situated in regions listed as less favoured areas, indicates significant development potential of goats in Romania. Significant investments in modernization and wider use of new technological procedure will raise productivity, improve welfare and quality of better monitoring the health of individual animals and goat herds and increase economic efficiency.

The health of a herd is assessed in relation to infectious diseases, parasitic and metabolic specific to goats.

Infectious diseases

Caprine arthritis-encephalitis (*Caprine Arthritis Encephalitis Virus* - CAEV) is a goat viral disease caused by a lentivirus belonging to the Family *Retroviridae*. The virus induces a persistent infection by incorporation of the CAEV genome into the DNA of host cell. The monocyte-macrophage cells are the main target of this virus. In clinical cases were described arthritis, mastitis, pneumonia, weight loss and encephalitis. Investigations on this infection in Romania were conducted by Gurău M. et al.,2015, in southeastern Romanian farm and by Mihai I. et al., 2018, in northeastern counties. The investigation results in southeastern farm revealed a high prevalence of CAEV-infection (38.46%), proved by serological investigation (active surveillance by ELISA-Ab exams), associated with low clinical cases of CAE, supporting the assertion that most of CAEV infected animals remains asymptomatic. The second study (Mihai I. et al., 2018) purpose was the detection of CAEV antibodies among goat populations from Vaslui County. During 2014-2016, blood samples were collected both from healthy animals and with clinical signs of disease. All serum samples were tested for CAEV antibody by agar gel immunodiffusion (AGID) test. The results of the investigation revealed a 31.86% (94 out of 295) seroprevalence.

A common infection of the respiratory tract of goats throughout the world is parainfluenza type 3 (PI-3). As with other respiratory viruses, PI-3 virus infection impairs the function of the alveolar macrophages and destroys cilia on the bronchial mucosa. This compromises the animal's natural clearance mechanism (defense mechanism) for removing pathogenic organisms from the lower respiratory tract making them susceptible to secondary bacterial infection particularly P. haemolytica infection. Uncomplicated PI-3 virus infection doesn't appear to be an important cause of death, but it may result in death because of bacterial pneumonia frequently in kids. In Romania Aniță et al., 2015, tested the immunodetection suitability of viral antigens in routinely fixed tissue specimens as a diagnostic tool for PI-3 infection in goats. Results of this study demonstrate that PI-3 infection should be considered as a possible cause of pneumonia in goats, along with respiratory syncytial virus and bacterial infections (*Mycoplasma* and *Mannhiemia spp*.).

A common infection of the respiratory tract of goats is *Pasteurella* infections result in pneumonia along with septicemia, arthritis, and otitis media. Spring outbreaks are more likely in kids 2 weeks to 2 months of age and frequently are seen in association with severe weather. Fall outbreaks are more likely to occur in 5- to 7-month-old goats after shipment to feedlots The bacteriological examination for *Pasteurella spp.* was performed for 149 samples collected from animals with or without respiratory clinical signs. Of these, 109 was positive for *Pasteurella spp.*, resulting in a prevalence of 73.15% and 40 samples (23.85%) with a negative result. Samples come from goats from Botoşani, Iaşi, Suceava and Vaslui counties, from Saanen, French Alpine, Carpathian and Saanen breeds, aged between 1.5 and 11, raised in extensive and intensive system, in free or permanent stabbing (Mihai I. et al, 2018). *Pasteurella* outbreaks are associated with morbidity rates of up to 50% of the flock or herd, but mortality rates typically are low (Plummer P.J. et al, 2012). *Pasteurella* infections frequently are secondary infections that follow an initial infection with one of several different viral or bacterial agents such as parainfluenza type 3, adenovirus type 6, respiratory syncytial virus, caprin herpesvirus 1, *Bordetella parapertussis* and *Mycoplasma ovipneumoniae (*Brogden Kim A.et al., 1998; Buddle B.M. et al., 1990).

Most of the infectious agents that cause respiratory disease are usually common inhabitants of the respiratory system. Of all goat maladies, those affecting the respiratory system can cause substantial loss through high morbidity and mortality (Bordeanu A.D. et al., 2012).

Researches on contagious ecthyma (Sore Mouth) in goats were described in many counties of Romania. Ecthyma is an infectious disease of sheep and goats, with acute evolution produced by an epitheliotrophic virus, clinically characterized by vesiculo - pustular eruption, with

predominantly buccal, podal, genital, mammary and ocular localizations. Clinically, the disease develops overactive in lambs and goes out, with deterioration in health, swelling of the nose, followed by a confluent and massive vesiculo - pustular eruption, on the lips and the mucous membrane, with regional adenopathy. Sometimes due to the spread of lesions on the respiratory and digestive mucosa, symptoms of ronchopneumonia or enteritis with a fatal outcome occur in 36-40 hours. This infectious disease caused by an epitheliotropic virus (ORF, family *Poxviridae*), has usually an acute evolution. The disease was first recoded in Romania by Riegler in 1935, near Bucharest. Latest epidemiological investigations in Romania (during 2008-2012) on Sore Mouth 490 were made by Rusu R.O., highlighting the circulation of ORF virus in goat herds from Iași and Botoșani Counties, using molecular methods for viral detection and characterization (Rusu RO et al., 2014). The same author studied the efficacy of vaccine protection (Scabivax) on goat herds: in kids and adults (Rusu RO et al., 2014). An outbreak of ectima was semnaleted in Suceava County at 50 adult goats and 20 kids, Saanen half-breed (Mihai I. et al, 2018).

Laminitis is a contagious disease with a polyfactorial etiology, characterized by a necrotic inflammation of the keratogenic membrane, followed in many cases by de-congestion. The main etiological agent is Dichelobacter (Bacteroides) nodosus, but from lesions are constantly isolated Fusobacterium necrophorum subsp. necrophorum and Arcanobacter pyogenes, and inconstantly Spirocheta penortha, Clostridium perfringens, Staphylococcus spp., Streptococcus spp., Dichelobacter nodosus (Velescu Elena, 1996). 59 cases of pododermatitis was diagnosticated in Vaslui county, Carpathian adult goats (Mihai I. et al., 2018). Goat hoof problems involve significant economic losses. Regular hoof adjustment and early diagnosis are the key to prevention and foot therapy. Lame goats spend more time lying down, eat less, milk production decreases and reproductive traits are less expressed. Once introduced into a herd, the disease spreads rapidly, most commonly affecting young animals up to one year of age. When the lameness is severe, the affected goats have a fever, are apathetic, with warm and painful coronary bands on the hooves. Foot injuries can become chronic if the initial phase is not diagnosed in time.

Another infectious disease prevalent in goats reared in small farms is gangrenous mastitis caused by *Staphylococcus aureus*. Romania pays special attention to development of livestock sector growth through the introduction of goat breeds with high milk production and genetic improvement of indigenous characters. For this purpose, there were created optimal conditions for maintenance and proper nutrition, and the genetic potential of animals is scientifically directed towards high yields of milk and meat. These, would greatly increase profitability if the morbidity and mortality due to udder disease were reduced. The main consequence of an incorrectly milking is mastitis. These are inflammation affecting the secretory epithelium, the lining of milk ducts and interstitial tissue. Microbial etiology of mastitis in goats includes a wide range of bacterial species, the most important being *Staphylococcus spp*. and *Mycoplasma Spp.* Gangrenous mastitis, caused by *Staphylococcus aureus*, is the most severe reported in goats, resulting in the animal's death or in incomplete or partial sloughing of the udder. In Romania, recent studies were made by Velescu et al., 2009 and Tudose A. et al., 2010 highlighting the importance of the early treatment of this infection. Tudose A. et al. 2010, also evaluated the immune response after vaccination against gangrenous mastitis, by dynamic research of serum protein fractions in goats in vaccinated and unvaccinated groups, revealing the increasing concentration of γ-globulins and decreasing of albumin levels.

Parasitic diseases

Is well known that goats are very sensitive to internal parasitism, which can cause a decrease in fertility, abortion, an increased susceptibility to diseases and death. In 2011, Iacob O.C.

conducted a study on 1450 Carpathian breed goats, in order to reveal different aspects of their digestive and pulmonary parasitogram. Following examinations, the author claims that Eimeria genus was dominant to all age categories, with an extensivity of 90- 100% and a value of OPG (oocysts per gram of faeces) between 0-2500. According to Iacob O.C. (2011), nematodes of the family Trichostrongylidae were dominant in adult goats, with an extensivity of 100% and an OPG value between 2000-5100, which defines a strong infestation; in young goats and bucks (treated against parasites two weeks previous to going out to pasture), trichostrongillids had an extensivity of 0-10% and a 0-50 OPG value. Pulmonary nematodes of the Protostrongylus genus and Protostrongylus rufescens species were dominant through unique infestation in adult goats and pregnant youth (Iacob O.C., 2011). Trematodes of the Dicrocoelium genus and Dicrocoelium lanceatum species were represented by rare invasional elements identified in young individuals and adult does (Iacob O.C., 2011). Finally, Iacob O.C. (2011) claims that a medical prophylaxis applied selectively in the herd according to age and short-term economic interest, contributes to the infestation and disease of animals at their first grazing, to the parasitical pollution of grazing areas and to the recording of much higher economical losses.

Metabolic disorders

Iodine deficiency determines the appearance of goiter, which is a characteristic of thyroid gland enlargement in all domestic mammals, birds and reptiles (Osame S. et al., 1994; Corradini P. et al., 2000; Garner M.M. et al., 2002; Fyfe J.C. et al., 2003). Sometimes, iodine deficiency during pregnancy could be the cause and the kids surviving after birth develop goiter in later stages of life (Vijlder D., 2003). Goiter in utero is caused by either primary or secondary iodine deficiency (Maxi M.G. et al., 2007). Among the incriminated cases in the appearance of kids goiter is the feeding of pregnant females with low iodine feed or goitrogenic compounds which interfere with thyroxinogenesis (brassica plants, soybean byproducts and water with high content of calcium and nitrates) (Blood D.C. et al., 2000; Radostits O.M. et al., 2007; Sing R. and Beigh S.A., 2013). The last outbreak reported in Romania regarding iodine deficiency in kids, was recorded by Mihai I. et al. (2018) within an Anglo Nubian goat farm. According to Mihai I. et al. (2018), the pregnant goats were fed with goitrogenic plants (cabbage), but there was no palpable enlargement of the dams thyroid glands. Clinically, iodine deficiency is characterized by the presence in the upper third of the neck of a submandibular bilobata formation that overlays the trachea just below the larynx, symmetrical, united in the distal part (Liklater & Smith, 1993). In order to reduce the economical wastage caused both the therapeutic expenses and an increased mortality rate, Mihai I. et al. (2018), recommended supplementing the iodized salt fodder ration as well as avoiding the goitrogenic feeding of goats in the last months of pregnancy.

The epidemiological situation in Romania has undergone significant changes in recent years, our country is the southeastern border of the European Union, which involves an increased risk for all major animal diseases and, of course, monitoring programs and greater control than in other Member States of the European Union.

The National Sanitary Veterinary and Food Safety Authority (ANSVSA), in accordance with European legislation, lays down rules and implementing measures for veterinary actions, which are included in the program for the surveillance, prevention, control and eradication of animal diseases, those transmitted by in humans, animal welfare and the environment, as well as the identification and registration of animals of the bovine, porcine, ovine, caprine and equine species. National programs include specifications on reportable diseases, maneuvers performed for clinical monitoring, serological surveillance (active/passive) and virological surveillance.

The list of reportable diseases in goats includes foot-and-mouth disease, anthrax, small ruminant plague, ovine and caprine smallpox, ovine and caprine brucellosis, *Brucella melitensis* infection, ovine brucellosis or *Brucella ovis* infection, campylobacteriosis, Maedi Visna, contagious agalaxia goat, pulmonary adenomatosis, communicable diseases, zoonoses and emerging diseases: bacterial (streptococcus, staphylococcus, colibacillosis) fungal (aspergillosis), viral (papillomatosis), parasitic (echinococcosis / hydatidosis, pneumocystosis, tetanus, sarcocystosis, skin caused by mites).

Passive serological surveillance includes taking samples to assess health status and monitoring tests for the suspected pathogen, samples may be: blood / serum, secretions / excretions, abortions, etc. The analyzes and laboratory examinations performed during the official control must be performed only with accredited methods in accordance with the provisions of art. 12 of Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure compliance with feed and food law, animal health and animal welfare, as subsequently amended.

The risk of introducing new diseases in Romania can be represented by: the evolution of diseases in the countries close to the Romanian borders; the unknown epidemiological status of these countries; - illegal crossing of borders with animals; the movement of persons and vehicles, in particular at the border.

On the Romanian territory, the risk can be represented by: the absence of biosecurity conditions on the farm; uncontrolled movements of animals; direct or indirect contact of dwellings with animals of unknown health.

The development of solutions for goat farmers in our country in the context of current challenges involves establishing the epidemiological status, health of goats and defining biosecurity measures to reduce the risks of introduction and spread of diseases in the herd.

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PRELIMINARY INVESTIGATIONS ON PREVALENCE OF ESBL-PRODUCTION *ESCHERICHIA COLI* STRAINS IN SWINE FROM BOTOȘANI COUNTY

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Abstract

Administration of antimicrobials to food-producing animals increases the risk of higher antimicrobial resistance in normal intestinal flora. The present preliminary study was conducted to investigate the presence of extended spectrum beta-lactamase (ESBL)-producing Escherichia coli strains in healthy swine from Botoșani County. During 2016-2018, a total of 87 samples of luminal contents of gut sections (cecal) were collected and tested. Fifty-one (51,72%) E. coli isolates were identified as ESBL-producing strains. These preliminary results reflect the selective pressure, caused by intense and less prudent use of the antimicrobials in swine production in our country. Moreover, commensal E. coli can be a reservoir for antimicrobial resistance genes, which can be transferred to pathogenic bacteria. Therefore, resistance genes transferring from farm to fork represent a public health emerging danger by the potential of producing difficult-to-treat pathogens.

Keywords: *Escherichia coli*, ESBL-producing strains, swine

Introduction

Antibiotic resistance in animals becomes a public health issue when there is transmission of antibiotic resistant bacteria, or their resistance genes, from animals to humans. The extensive use of antimicrobials in food-producing animals has potential to raise antimicrobial-resistance in enteric commensal bacteria. These bacteria may constitute a significant reservoir of antibiotic resistance determinants, which can be transferred to pathogenic bacteria for humans and animals. Food contamination with antimicrobial-resistant bacteria is a public health concern because the resistant organisms can be transferred to humans through the consumption of contaminated food and can thus compromise human health (EFSA/ECDC, 2017).

The antimicrobial agents can be divided into several groups, three of which are used in veterinary medicine, namely, penicillin, first to fourth-generation cephalosporins and β-lactamase inhibitors (Geser et al, 2011). Beta-lactam antimicrobials are one of the important antimicrobial agents in veterinary medicine respectively in swine production. β-lactamases are enzymes that break down β-lactam antibiotics, which constitute the biggest class of antibiotics. Resistance to this class of antibiotics, mediated by extended-spectrum β-lactamases (ESBL) has been increasingly reported (van Damme et al, 2017). Excessive use of extended-spectrum β-lactam antibiotics, mainly the 3rd generation cephalosporins, leads to the production of ESBLs amongst Gram negative rods of the *Enterobacteriaceae* family, such as *Escherichia coli.* ESBL enzymes break down penicillin, amino-, ureido-, and carboxypenicillins, 1st, 2nd, and 3rd generation cephalosporins. Carbapenems are the only β-lactam antibiotics that have an effective functioning on ESBL-producing bacteria.

Escherichia coli are highly adapted organisms that live in the gastrointestinal tract of both animals and humans and survive in environment (water, soil or fecal matter). The presence of *E. coli* can indicate fecal contamination in the environment and can play an integral part in monitoring the transmission of antimicrobial resistance genes within bacterial populations (Hansen et al., 2013). The horizontal transfer of resistance genes, especially mobile genetic elements, has led to

the rapid emergence of multidrug-resistant *E. coli* in the swine production, which may be delivered to humans (Collignon et al, 2007).

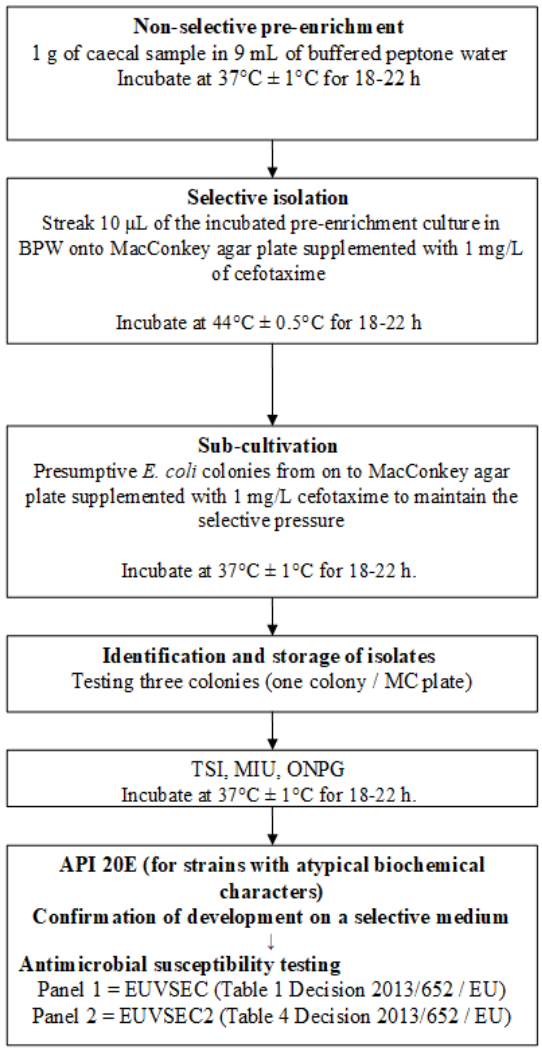
Material and methods

During 2016-2018, 87 samples of luminal contents of gut sections (cecal) were collected from slaughtered swine. All pigs originated from farms in Botoșani County (table 1). The laboratory investigations were carried out at the Sanitary Veterinary and Food Safety Laboratory Iaşi and at Microbiology Laboratory from University of Agricultural Sciences and Veterinary Medicine Iași.

Table 1

Sampling distribution during research period

Year	*2016*	*2017*	*2018*
No. of tested samples	35	23	29



The samples were collected randomly from slaughtered pigs. Sampling was made in sterile, intact bags, without being exposed to extreme temperatures, then transported to the lab. All samples were submitted to laboratory testing in the shortest time, respectively in maximum 48 hours after sampling.

Isolation of ESBL -producing *E. coli* strains from swine cecal samples was performed according to the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) protocol (Fig. 1).

The isolation protocol for samples of luminal contents of gut sections (cecal) consists in use of a pre-enrichment step. Thus, following aseptic opening of the cecum, the content was aspirated with a sterile pipette and then released in a sterile Falcon tube, to which was added 9 ml buffered peptone water (Oxoid, Basingstoke, UK).

Fig. 1 Schematic representation of protocol used for ESBL producing *E. coli* detection and taxonomic classification of the isolates (EURL-AR)

The tubes were incubated with lightly threaded plugs under aerobic conditions at 37 ± 1 ° C. After 20 ± 2 hours of incubation, samples were transplanted into MacConkey agar (Oxoid, Basingstoke, UK), supplemented with cefotaxime (CTX) (Sigma-Aldrich, US), a selective medium recommended for the detection of ESBL-producing *Escherichia coli* strains. After 24 hours of incubation at 44°C, the plates were examined and three presumptive cephalosporinase-producing colonies were transplanted into Petri dishes with cepotaxime-supplemented MacConkey (CTX) medium (Sigma-Aldrich, US) (fig.2). After another 24 hours of incubation at 37 ± 1 ° C, *Escherichia coli*-type colonies were examined and identified. For bacterial species confirmation, the isolated strains were cultured on MIU (mobility, indole, urea) / TSI (glucose, lactose, hydrogen sulfide, gas) polytropic media (Oxoid, Basingstoke, UK) (fig.3).

Fig. 2 *Escherichia coli* colonies presumably synthesizing ESBL on MacConkey agar (Oxoid, UK) supplemented with cefotaxime (Sigma-Aldrich, US)

Fig. 3 *Escherichia coli* confirmation on MIU/TSI media (Oxoid, UK)

Broth microdilution was performed forward for antimicrobial susceptibility testing of *Escherichia coli* isolates. For this purpose, in the first step was used *Sensititre E. coli* EUVSEC plate (TrekDiagnostic System, Thermofisher) and the protocol recommended by the producer. Minimum inhibitory concentrations (MIC) (mg/l) were determined for the following antibiotics: sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), tetracycline (TET), meropenem (MERO), azithromycin (AZI), nalidixic acid (NAL), cefotaxime (FOT), chloramphenicol (CHL), tigecycline (TGC), ceftazidime (TAZ), colistin (COL), ampicillin (AMP) and gentamicin (GEN). For each plate was used a positive control.

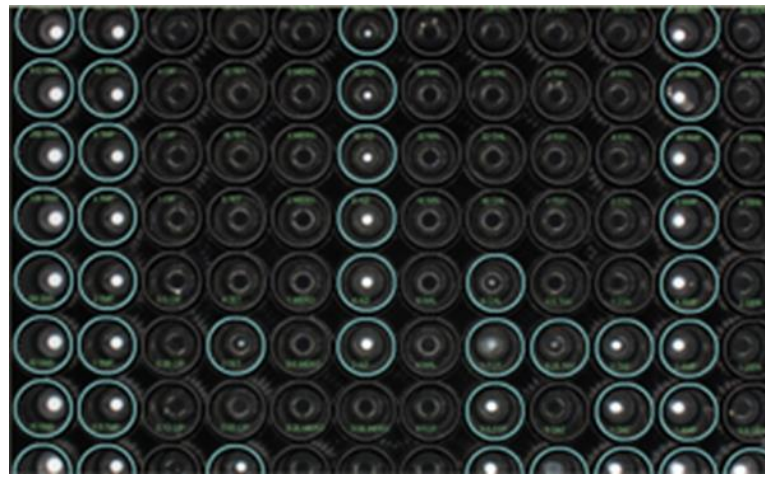


Fig. 4. *Sensititre E. coli* EUVSEC plate (TrekDiagnostic System, Thermofisher)

Beta-lactamase *E. coli* spectrum isolates identified as resistant to cefotaxime and/or ceftazidime and/or meropenem, were subsequently tested with a second antimicrobial panel *Sensititre E. coli* EUVSEC2 plate (TrekDiagnostic System, Thermofisher) necessary for phenotypic verification of presumably carbapenemase-producing *E. coli* strains. Minimum inhibitory concentrations (MIC) (mg/l) were determined for the following antibiotics: cefoxitin (FOX), ertapenem (ETP), imipenem (IMI), meropenem (MERO), ceftazidime (TAZ), cefepime (FEP), cefotaxime/clavulanic acid (F/C), ceftazidime / clavulanic acid (T/C), Cefotaxime (TRM), temocillin (TRM).

Results and discussions

Swine is one of the major food-producing animals in several European countries including Romania. More important, swine has been implicated as a source of antimicrobial-resistant bacteria indicating the importance of identification of antimicrobial resistance in food-producing animals. Although, data on the occurrence of ESBL-producing *E. coli* strains from healthy swine are very limited in Romania. In this study, we screened for ESBL-producing *Escherichia coli* in swine luminal contents of gut sections (cecal) from Botoșani County. The results highlighted that 45 out of 87 isolates were positive for ESBL-producing *E. coli* (Fig. 5).

Using broth microdilution method for certain antibiotics such as cefotaxime and ceftazidime, testing was performed using two different panel plates. Although most ESBL-producing *E. coli strains* have resistance to both compounds, some ESBL enzymes primarily confer resistance to only one of the compounds. Confirmatory synergy testing was also provided so that the ESBL phenotype could be identified. Cefoxitin was also included to identify the AmpC phenotype. Antibiotics, meropenem, imipenem and ertapenem were included to identify suspected carbapenemase-producing *E. coli* strains. The effectiveness of temocillin (6-α-methoxy-ticarcillin) is not affected by most ESBL and AmpC enzymes and this antibiotic may be especially useful in human medicine to treat urinary tract infections caused by gram-negative organisms producing ESBL (Oteo, 2008 ; Livermore and Tulkens, 2009). Temocillin susceptibility allows for additional phenotypic characterization of carbapenemase-producing *E. coli* strains.

The aim of the study, by using both panel testing, was to deduce the ESBL-producing *E. coli* strains that are responsible for conferring the phenotypic profile of resistance to third-generation cephalosporins or meropenem, providing additional important epidemiological information.

If we analyze to the total number of confirmed isolates, the highest prevalence was reported in 2016 representing 57,14% (20 out of 35 samples), followed by 2017 with a percentage of 56,52% (13 out of 23 samples). The lowest prevalence of ESBL-producing *Escherichia coli* was registered in 2018 representing 41,37% (12 out of 29 samples) observing a slightly decreasing of the number of ESBL-producing *E. coli* samples.

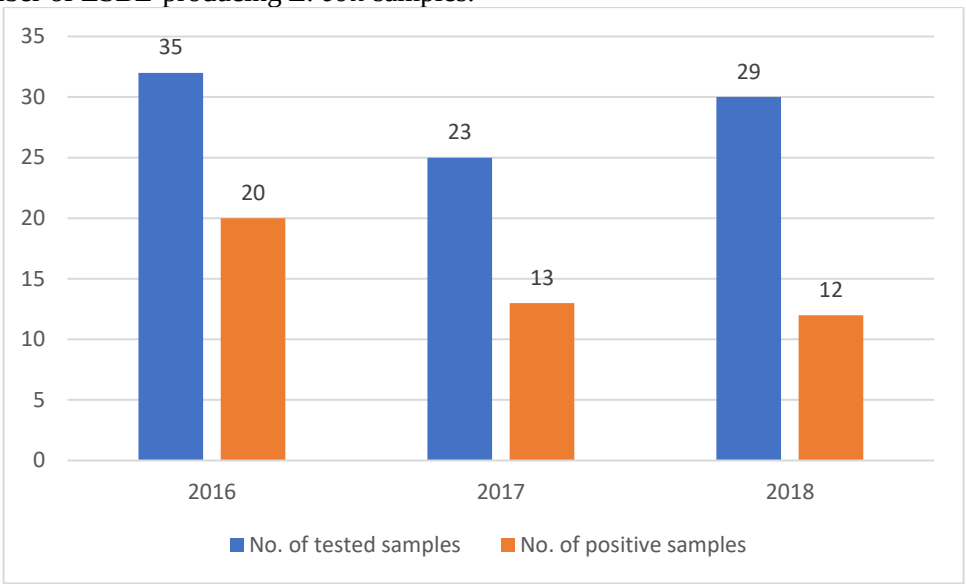


Fig. 5 Distribution of the positive ESBL-producing *Escherichia coli* identified in swine from Botoşani County

In Romania, a similar study published by Miliță et al. revealed that in 2015 the prevalence of ESBL/AmpC producing *E. coli* isolates in swine cecal samples was 65,78% (223 out of 339 samples). Based on MIC obtained for 21 antimicrobials, the selected 223 strains were resistant to cefotaxime (100%), ampicillin (100%), cefepime (91.93%), ceftazidime (90.13%), sulfamethoxazole (73.54%), tetracycline (71.30%), trimethoprim (62.33%), ciprofloxacin (53.81%), chloramphenicol (41.70%), nalidixic acid (39.91%), gentamicin (20.63%), cefoxitin (20.63%), azithromycin (14,35%) and colistin (3.59%) (Miliță et al, 2017).

ESBL-producing Enterobacteriaceae are reported in livestock, companion animals and wildlife. In the European program for monitoring antimicrobial resistance, cefotaxime-resistance was shown among *E. coli* isolates randomly selected from broilers at the slaughterhouse (6.6%), from pigs (1.3%), and from cattle (1.2%) (EFSA/ECDC, 2015).

In 2017, the overall prevalence of presumptive ESBL producing *E. coli* in fattening pigs in the EU was 30.62%, that is slightly higher than reported in 2015 (30.2%). Within Eastern European countries, the data collected in 2017 revealed that Hungary had the highest prevalence rate (56.2%), while in 2015 Bulgaria was the country with the highest one (49.8%). The lowest rates in 2017 were registered in the Czech Republic (Bergšpica et al., 2020).

The transmission and spread of infectious diseases through population mobility, including multidrug-resistant organisms such as *Escherichia coli* that produce extended-spectrum- β lactamases is an emerging phenomenon. In Europe from 2010 to 2013, a 9.5% to 12.6% significant increase in resistance to third-generation cephalosporins in *E. coli*, which is an indicator for ESBL-production, was observed in invasive human isolates (Karanika S. et al, 2016). Antimicrobial resistance in the EU/EEA published in the Annual epidemiological report for 2019 that the most

commonly reported bacterial species was *Escherichia coli* (44.2%). Moreover, more than half of the *E. coli* isolates reported in 2019 were resistant to at least one antimicrobial group under surveillance, and combined resistance to several antimicrobial groups was frequent (EARS-Net, 2020).

Humans and swine may be exposed to antibiotic resistant bacteria by direct physical contact or indirect contact via the environment, that is fomites and the natural environment. Exposure to ESBL-producing *E. coli* could lead to infection, carriage or disease in both humans and pigs. Studies looking at transmission of β-lactamase-producing *Enterobacteriaceae* between humans and livestock farms are limited to pig farms, but results suggest that working with positive pigs is associated with an increased risk of carriage (Dohmen *at al*., 2015). Moreover, at slaughterhouses, a risk of cross-contamination of meat exists, especially during evisceration, where carcasses can be contaminated by AMR bacteria from the fecal content of the same or different pigs (Wu et al, 2009). Food processing environments are considered to be important intermediate reservoirs and vectors of multi resistance bacteria, and also food handlers pose a risk of transmission of ESBL producing bacteria (Oniciuc et al, 2019).

Conclusions

Antimicrobial resistance poses a growing threat to public health. Overall, the prevalence of ESBL producing *E. coli* isolated from pigs is increasing in Europe over the years. The information published the literature highlights the urgent need to reconsider the responsible use of antimicrobials at farm level. Moreover, the antimicrobial resistance surveillance should provide up-to-date and relevant information to monitor the appropriateness of therapy guidelines, public health interventions, infection control policies, and antimicrobial development.

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PRELIMINARY RESULTS REGARDING THE PREVALENCE OF CTX-M GENES IDENTIFIED IN *E. COLI* STRAINS ISOLATED FROM SLAUGHTERED PIGS

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Abstract

Extended spectrum beta-lactamase (ESBL)-producing enterobacteriaceae and AmpC cephalosporinases are of major importance for public health because these bacteria have low sensitivity to antibiotics such as extended spectrum cephalosporins, which are antimicrobials widely used both in human and in veterinary medicine. Such strains, especially Escherichia coli (E. coli), have been frequently isolated from pigs too, production animals being considered carriers with major implications in the transmission chain of these strains in humans. The aim of this study was to characterise the molecular substrate of ESBL-positive E. coli strains isolated from slaughtered pigs from 3 slaughter houses from the Moldova area by identifying the CTX-M genes. After collection, the samples were primarily processed for phenotypical identification and confirmation of ESBL-positive E. coli strains. Bacterial DNA extraction for the target strains was carried out using the "boiled preps" method. Identification of the bla$_{CTX-M}$ (bla$_{CTX-M-9}$; bla$_{CTX-M-1}$) genes was carried out by PCR using the specific protocol. Molecular investigations revealed that out of the 118 analysed samples, the bla$_{CTX-M-U}$ gene was identified in 61% (72/118). Characterisation of the CTX-M groups signalled the presence of the CTX-M-1 group in 44/72 (61.11%) of the analysed strains, and the presence of the CTX-M-9 group in 18/72 (25%) of the strains. This study emphasised a high prevalence of CTX-M enzyme-producing E. coli strains isolated from the caecum of slaughtered pigs.

Key words: *E. coli, CTX-M, slaughtered pigs*

Antimicrobial resistance (AMR) is a subject of global interest, with special implications for public health, especially in terms of complicating the treatment of bacterial infections. The occurrence and spread of AMR was assigned to wrong or excessive use of antibiotics both in human and in veterinary medicine.

Along with intensive use of antibiotics in veterinary medicine, the degree of antimicrobial resistance in production animals followed an ascending trend since the first reported cases of AMR. Also, it has been proven that animal farms and wastewater treatment plants of slaughter houses contain a more diverse set of plasmids and genetic cassettes, compared to the wastewater from hospitals, which means that the formers could be considered a hot point for horizontal transfer of antimicrobial resistance genes (Yuan W. *et al.*, 2020).

Production animals, such as poultry or pigs, which are carriers of ESBL-positive Enterobacteriaceae, even without showing clinical signs of disease, are possible reservoirs of ESBL enzymes that can be transferred to humans either through the food chain, or by improper handling and improper cooking of meat (Carattoli A., 2008). The pig slaughtering process includes several stages; some of them ensure decrease of microbial contaminants, but others, such as the evisceration stage, increase the risk of contamination (Warriner K. *et al.*, 2002 Wu S. *et al.*, 2009). Consequently, faecal carriage with ESBL-producing *E. coli* strains in pigs is of special importance through the transfer of these strains from the intestines of animals to meat during slaughtering.

The number of reported ESBL enzymes is continuously increasing (Ur Rahman S. *et al.*, 2018); however, the most common remain those that belong to the CTX-M group (Peirano G. *et al.*, 2019). In production animals, in the 1990s, the most frequently identified ESBL genes were *bla*$_{TEM}$ and *bla*$_{SHV}$, but nowadays the most common are *bla*$_{CTX-M}$ (Hawkey P.M. *et al.*, 2009; Irrgang

A. *et a1*., 2018). There are hundreds of versions of the *bla*$_{CTX-M}$ genes identified, and they have a more intense activity against cephalosporins, such as cefotaxime, than against other oximino-β-lactams (Blair J.M.A *et al*., 2015).

The aim of our study was to characterise the molecular substrate and to determine the prevalence of CTX-M genes in *E. coli* strains isolated from the caecum of pigs slaughtered in three slaughter houses from the Moldova region.

Material and method

The work protocol for the caecum samples and stool cultures was first aimed at opening the caecum in an aseptic manner and collecting 1 ± 0.1 g from the content. After this stage, the sample of caecal content was pre-enriched by using buffered peptone water (Oxoid, Basingstoke, United Kingdom). The screening of ESBL-producing *E. coli* strains was carried out by cultivation on the MacConkey medium (Oxoid, Basingstoke, United Kingdom) with an addition of cefotaxime (CTX) (Sigma-Aldrich). The colonies that showed morphology characteristic of *E. coli* strains were confirmed based on the chemical properties using the media TSI, MIU and API 20E. Phenotypical confirmation of the production of ESBL enzymes was carried out through the microdilution in broth method, using the EUVSEC2 plates that contain cefoxitine, cefepime, cefotaxime, ceftazidime and clavulanic acid combined with cefotaxime or ceftazidime. The results were interpreted based on the synergy tests between the clavulanic acid and cefotaxime or ceftazidime. After phenotypical processing of the obtained isolates, bacterial DNA extraction was carried out through the "boiled preps" technique. Molecular characterisation of strains first aimed at identifying the *bla*$_{CTX-M}$ genes and then at identifying the genes that encode two of the most common groups of CTX-M enzymes: CTX-M-1 and CTX-M-9. The molecular investigations were carried out by PCR while observing the protocol recommended by Wedley *et. al*. The patterns of nucleotide sequences, length and alignment temperature for each target gene are indicated in *table 1*.

Table 1

Primers used for the identification of genes that encode the CTX-M enzymes

Primers	Nucleotide sequences (5'-3')	Length (bp)	Alignment temperature	Reference
CTX-Mu	Fw: 5'-ATGTGCAGYACCAGTAARGTKATGGC-3' Rev: 5'-TGGGTRAARTARGTSACCAGAAYCAGCGG-3'	593	58°C	Wedley A.L. et al., 2011
CTX-M-1	Fw: 5'-ATGGTTAAAAAATCACTGCG-3' Rev: 5'- TTACAAACCGTCGGTGAC-3'	876		
CTX-M-9	Fw:5'- ATGGTGACAAAGAGAGTGCAAC-3' Rev: 5'- TTACAGCCCTTCGGCGATG-3'	876		

Results and discussions

118 strains of presumptive ESBL enzyme-producing *Escherichia coli* strains were analysed from a molecular point of view. The conducted investigations highlighted the presence of the *bla*$_{CTX-M-U}$ gene in 72/118 (61%) of the strains. Characterisation of the CTX-M groups in the strains taken under study has signalled the presence of the CTX-M-1 group in 44/72 (61.11%) of the isolates, which was the dominating group, while the CTX-M-9 group was identified in 18/72

(25%) of the strains. Furthermore, 10/72 (13.89%) strains of animal origin with positive signal for the *bla*$_{\text{CTX-M-U}}$ gene did not belong to any of the two aforementioned groups (*figure 1*).

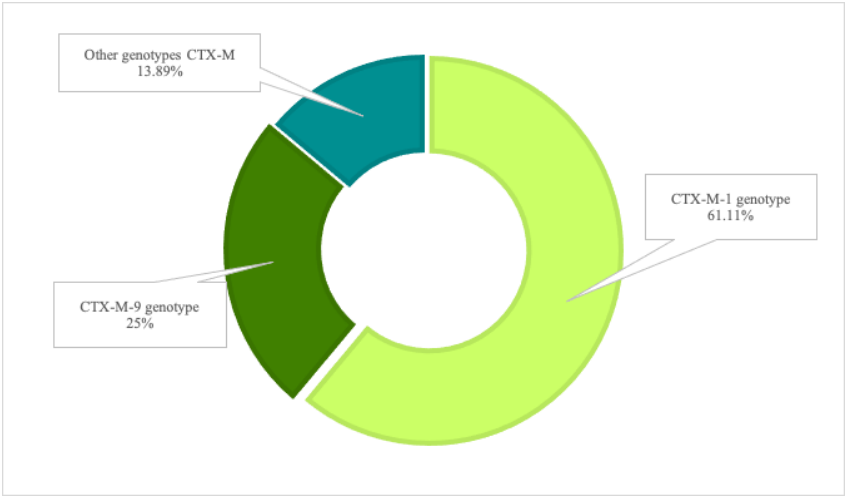

Figure 1 Prevalence of CTX-M genotypes identified in the analysed *E. coli* strains

According to the Commission Implementing Decision 2013/652/EU, in the period 2015 and 2017, within the routine AMR monitoring program, the EFSA collected information about the prevalence of *E. coli* strains resistant to antibiotics isolated from the caecum of pigs and from pork (European Food Safety Authority; European Centre for Disease Prevention and Control, 2019). In 2017, in the European Union (EU), the prevalence of ESBL-producing *E. coli* strains isolated from the caecum of pigs was 30.62%, lower than the one reported in 2015 (30.2%) (European Food Safety Authority; European Centre for Disease Prevention and Control, 2019). In regard to East European countries, in 2017, Hungary had the highest prevalence rate (56.2%), while in 2015, Bulgaria was the country with the highest prevalence (49.8%) (European Food Safety Authority; European Centre for Disease Prevention and Control, 2017; European Food Safety Authority; European Centre for Disease Prevention and Control, 2019). In addition, within the same routine AMR monitoring program, Romania reported in 2015 a prevalence of 46.6% of ESBL-positive *E. coli* strains isolated from the caecum of pigs, and in 2017, a prevalence of 53.7%, being the second country, immediately after Hungary, with a high prevalence in East Europe. The high prevalence of ESBL-producing *E. coli* strains isolated from pigs obtained in this study can be correlated with a high consumption of antibiotics of the type of penicillin or third-generation or fourth-generation cephalosporins in the industry of production animals; the highest sales for the aforementioned antibiotics were registered in East and South Europe (European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption, 2019; Ieva B *et al.*, 2020). Moreover, the fact that screening was carried out on samples collected from the caecal content, which is a natural habitat for the *E. coli* strains, should be taken into consideration.

The prevalence of ESBL-positive *E. coli* strains in fattening pigs (studies conducted on samples of caecal content) varies significantly in the EU depending on the countries. In general, the prevalence of ESBL-producing *E. coli* strains isolated from pigs has seen an ascending trend along the years.

At a worldwide level, strategies to solve the global problem of AMR have been discussed and will basically also be relevant in the battle against the spread of ESBL enzyme-producing

bacteria in the industry of production animals. For example, the information in the specialty literature recommends special attention to the principles of use of antibiotics, closer monitoring both of the consumption of antibiotics and of the antimicrobial resistance, as well as establishing preventive measures to diminish the risk of introduction or spread of AMR bacteria at the farm level. Moreover, faecal carriage with ESBL-positive enterobacteria represents a risk for the staff working in farms or in slaughter houses by possible intestinal colonisation with AMR pathogenic bacteria. In addition, pork can be cross-contaminated either during the slaughtering process, or by the staff.

Conclusions

This study emphasised a high prevalence of CTX-M enzyme-producing *E. coli* strains isolated from the caecum of slaughtered pigs. Furthermore, monitoring and rational use measures of antibiotics in farms are necessary.

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The surgical management of a diaphragmatic hernia in a cat – case report

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Abstract
A diaphragmatic hernia is an internal hernia, characterized by a defect of the musculotendinous plate between the thoracic and abdominal cavities, which allows the abdominal content to protrude into the thoracic cavity. This case study emphasizes the importance of choosing the propitious time in the surgical repair and also the anaesthesia principles in diaphragmatic herniorrhaphy. A 2-year-old intact female cat was referred with the following symptoms: open-mouth breathing, tachypnea, tachycardia, pale mucous membranes and a dyspnoea increasing when changing position. The definitive diagnosis was confirmed through thoracic and abdominal radiography and the surgical procedure was performed after the patient's condition was stable.
Key words: *diaphragmatic hernia, surgical repair, anaesthesia*

Introduction

A diaphragmatic hernia, congenital or acquired, develops when the continuity of the diaphragm is disrupted and the abdominal contents protrude into the thoracic cavity (Fossum, T. W. et al., 2013). Congenital or pleuroperitoneal hernia are seldom seen in dogs and cats because the affected animals usually die at birth or shortly thereafter (Tobias, K.M., 2017; Monnet, E., 2013). The most common diaphragmatic ruptures are those resulting from severe changes in abdominal and thoracic pressures, usually after blunt trauma, particularly motor vehicle accidents (Fossum, T. W. et al., 2013). The defect leads to a life-threatening respiratory condition and a potential entrapment of abdominal organs (Gibson, T. W. et al., 2005).

The most common clinical signs are dyspnea and vomiting, but the physical examination findings may vary acording to hernia origin (cronic or recent), animal age, concurrent disorders, type of viscera involved or the size and location of the diaphragmatic defect, such that nonspecific signs such as lethargy, anorexia and weight loss are seen. (Besalti, O. et al., 2011; Fossum, T. W. et al., 2013; Monnet, E., 2013; OZER, KURSAT, et al., 2007)

The treatment of choice is the surgical repair, but there is some controversy over timing of surgical intervention. One study suggest that mortality rate is higher in patients which had the surgery performed in the first 24 hours after the accident, or more than one year (Boudrieau et al. 1987), while a more recent one reported that dogs and cats that underwent surgery within 24 hours of admission had a good perioperative survival rate (Gibson, T. W. et al., 2005). Patients with overt symptoms of congenital diaphragmatic hernia are good candidates for surgical herniorraphy, but those that have mild or no clinical signs can receive a conservative treatment (Tobias, K.M., 2017; Reimer, S. B. et al., 2004). Another factor influencing the outcome of the procedure is representend by the anesthetic protocol, because of the animal's already compromised ventilation. Drugs with minimal respiratory depressant effect are recommended. Before and also during induction, oxygen should be provided to improve myocardial oxygenation; during maintenance variation of pressure ventilation has to be done in order to avoid apneea or reexpansion pulmonary edema. (Tobias, K.M., 2017; Fossum, T. W. et al., 2013).

Materials and methods

The study was conducted on a 2-year-old female cat, mixed breed, unspayed, 2,5 kg with acute onset of dyspnea, elevated heart rate, open-mouth breathing and pale oral and conjunctival mucosa. The cat resided both indoors and outdoors, thus recent trauma could not be ruled out.

Although haematology, biochemistry and urinalysis were unremarkable a slight elevation in liver enziymes was observed. The radiological exam revealed an indistinct diaphragmatic line and unusual soft tissue densities within the pleural cavity. Following the presumptive diagnosis of diaphragmatic hernia the patient was prepared for surgical correction of the defect.

The patient was premedicated with methadone at a dose rate of 0,3 mg/kg intravenously and the induction was made with propofol at a dose rate of 6 mg/kg intravenously. Maintenance was performed with isoflurane (2,5%).

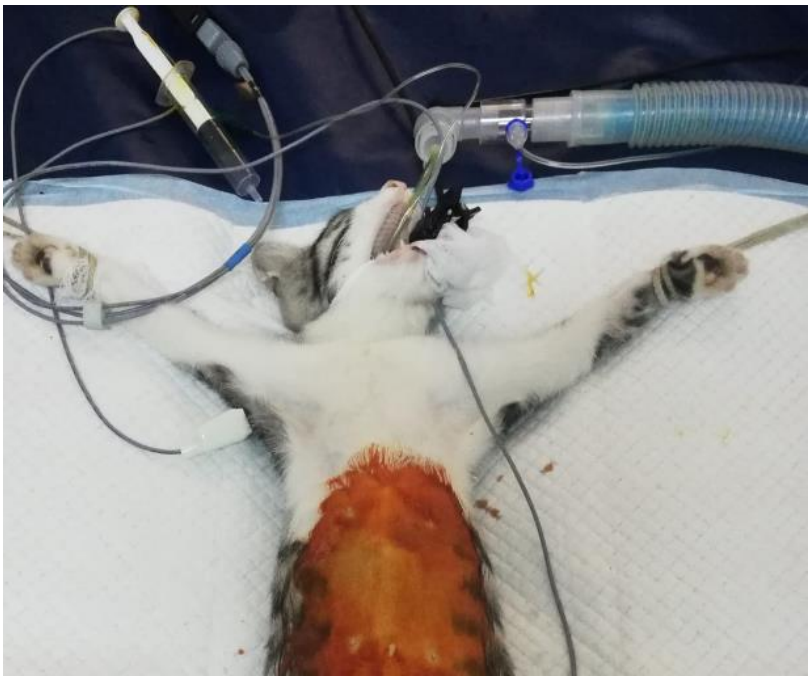

Figure 1. Preoperative management

The cat was placed in dorsal recumbency (Fig. 1) and the abdominal area was aseptically prepared for the surgical intervention (Fig. 2). The surgical approach includes a ventral median incision of the skin and muscular layer. Preceding this, the high-pressure ventilation which was previously induced, has to be immediately reduced in order to avoid rapid lung expansion.

Results

Once the abdominal cavity was opened (Fig. 3), the hernia site was identified and the protruded viscera were retracted smoothly into the abdomen as a result of the lack of adhesions (Fig. 4). The herniated organs were represented by the liver and the intestinal ansae. The organs were carefully examined for ischemia or other lesions and no damage was noticed.

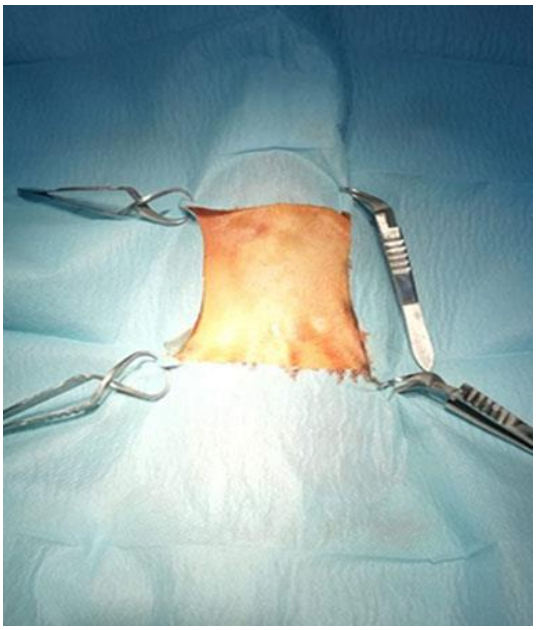

Figure 2. Preparing the surgical area

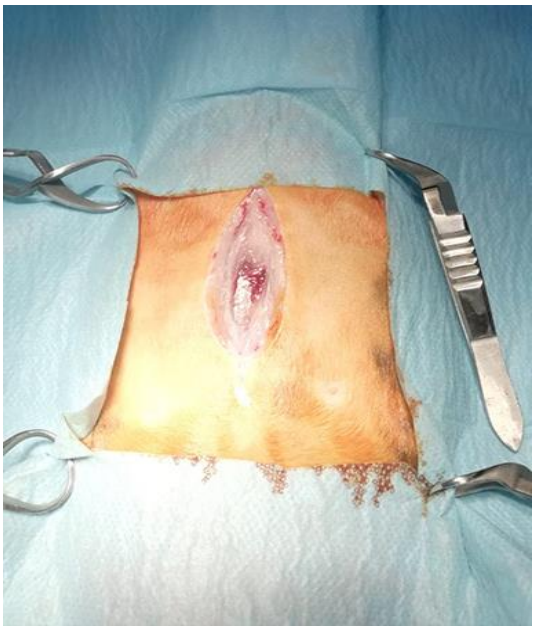

Figure 3. Skin incision

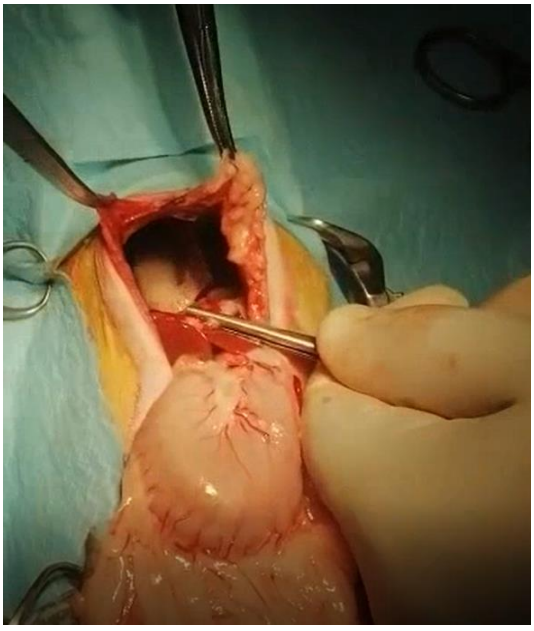

Figure 4. Hernia ring

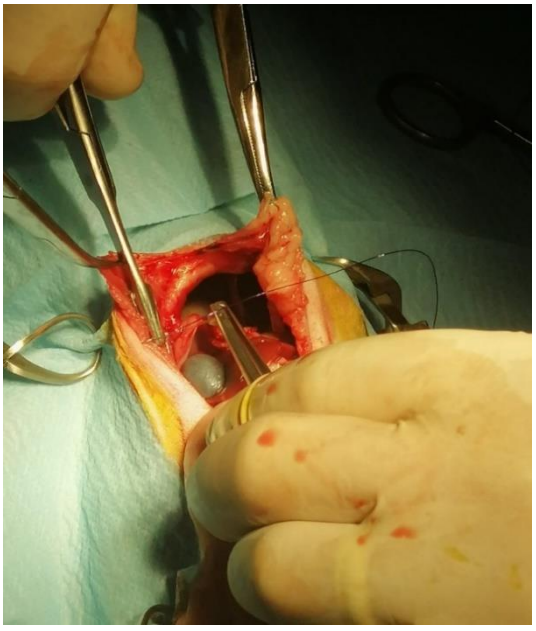

Figure 5. Hernia defect closure

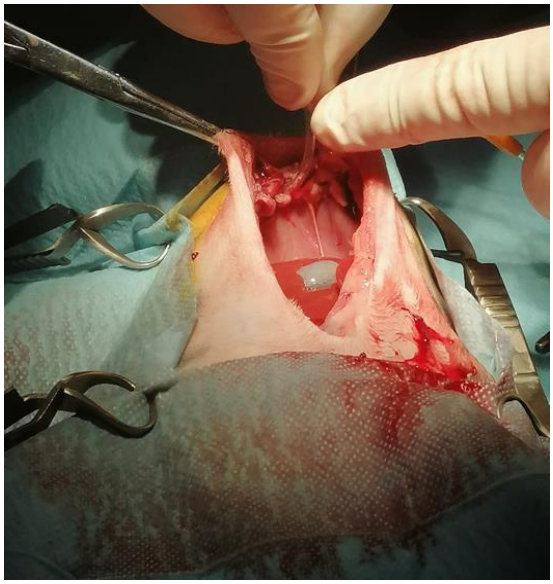

Figure 6. Pleural air removal

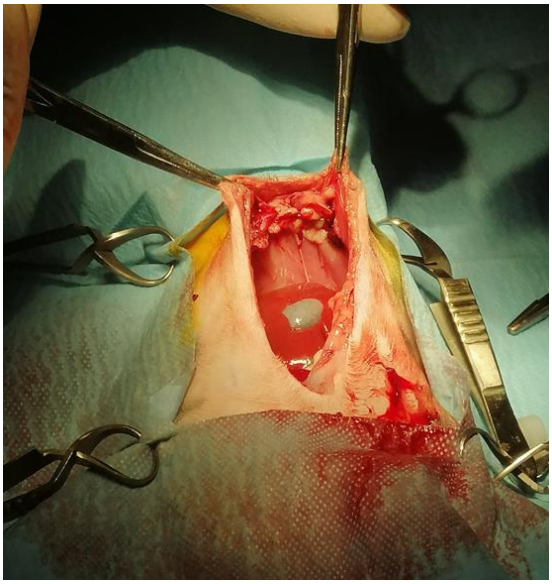

Figure 7. Inspecting the diaphragm integrity

The diaphragmatic defect is closed in a simple continuous suture pattern using an absorbable suture material (Fig. 5). Before placing the last suture, a fenestrated catheter attached to a three-way stopcock, must be used in order to remove the air from the pleural cavity (Fig. 6). The diaphragm surface and the abdominal cavity are explored for other associated injuries, but no lesions are found (Fig. 7). The abdominal incision is closed routinely in a multiple layer closure, using a continuous suture for the muscular layer and a simple innterrupted one for the skin.

Figure 8. Postoperative management

Discussions

Different factors may influence whether to follow a conservative treatment or to proceed with surgical reconstruction. There are records which show that occasionally, when dealing with a very wide defect linked with a poor condition of the patient, both types of treatment might not be

successful and the animal has to be euthanized (Keep, J. M., 1950). In more recent studies, for hernia rings that cannot be completey closed, patching has been reported using omentum, muscle, polypropylene mesh and also silicon rubber sheeting (Hunt GB, et al., 2013). On the contrary, in other cases the enlargement of the hernia defect has to be performed in order to remove incarcerated viscera that can not be reduced through gentle traction. (Monnet, E., 2013)

Patients undergoing diaphragmatic herniorraphy should be carefully monitored because acute ventilatory compromise may occur. The patient in this case had a cooperative behaviour and easily tolerated the face mask, therefore, it was pre-oxygenated for about 3 minutes prior to the induction of anaesthesia. Because this wasn't a long-standing case, the adhesions weren't present and even though the defect was large, the patient recovered uneventfully. To avoid hypoventilation and secundary hypoxia, the patient was maintained after surgery in an intensive care unit connected to an oxygen concentrator (Fig. 8).

Postoperative mortality rate varies from 10% (Tobias, K.M., 2017), 14% (Minihan, A. C., et al, 2004), 17,6 % (Schmiedt, C. W., et al., 2003) to 48 % (Fossum, T. W. et al., 2013). The prognosis is usually excelent if the patient survives the first 24 hours postoperatively. Schmiedt, C. W., (2003) highlights in a retrospective study that this impact factor is significantly associated with concurent injuries, while Thomas W. G. Gibson found in his retrospective study on 92 cases that timing of surgical intervention is also important, the early surgical intervention being associated with good perioperative survival rates.

Conclusions

Diaphragmatic hernias are associated with a semnificative respiratory embarressment, especially those of traumatic origin, which worsens the already modified condition caused by the morphological changes of the herniated organs. In conclusion the succeess of this type of surgery is increased by following the appropiate anesthetic protocol; with an adequate surgical tehnique and postoperative care, the prognosis of the surgery should be excellent.

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