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MILK FEVER AND RELATED POSTPARTUM DISEASES IN DAIRY CATTLE – A REVIEW

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Abstract

Milk fever is a metabolic condition that occurs in dairy cattle before or immediately after parturition as a result of low calcium (Ca^{++}) levels in the blood (hypocalcaemia). Based on its pathological changes, milk fever (hypocalcaemia) can be clinical or subclinical, being particularly prevalent in high-producing cows during the calving period.
The most common factors leading to milk fever include milk production, breed, parity, age, body condition score (BCS), and the composition of the cow's diet. The economic effects of milk fever are represented by decreases in milk production and fertility, finally resulting in culling of high-producing dairy cattle within herds. In order to establish the diagnosis of milk fever in dairy cattle, clinical and paraclinical examinations are used.
Milk fever prevention is economically essential for dairy farmers since it helps them avoid production and culling losses, and also increased veterinary expenses related with this condition. Numerous approaches have been introduced in order to mitigate hypocalcaemia, which include anionic salt feeding, low-calcium diets, vitamin D supplements, magnesium supplements, and peripartum body condition management. As a final conclusion, preventing milk fever is crucial for overcoming disease's economic impact on the dairy industry.

Key words: milk fever, dairy cattle, reproduction, prevention.

INTRODUCTION

As known, milk fever is among the most prevalent mineral-related metabolic disorders of dairy cows, occurring just before or shortly after parturition as a result of excessive calcium loss from the blood (50 g per day), in order to ensure rapid milk synthesis (DeGaris and Lean, 2008; Thirunavukkarasu et al., 2010; Khan A. et. al., 2012).
Known also as periparturient hypocalcaemia or periparturient paresis, milk fever is defined by Horst et al. (2005) cited by Pacheco H.A. et al. (2018), as a metabolic condition, affecting dairy cows around parturition.

MILK FEVER KEY HIGHLIGHTS

This is one of the metabolic disorders that most commonly occurs in adult cows within 48 hours of parturition, but can also occur weeks before or after it. Generally, dairy cows produce 10 liters or more of colostrum, containing 23g or more of calcium on the day of parturition, which is approximately 6 times as much calcium as the extracellular calcium pool includes (Aberaw A., 2017).

According to Reinhardt T. et al. (2011), after calving, blood calcium concentrations in about half of dairy cattle in their second lactation and above, drop below the threshold for subclinical hypocalcaemia. Therefore, metabolic diseases are caused by the animals' inability to cope with the metabolic requirements of high milk production, and their etiology can be linked directly to insults encountered during the transition period.

Negative energy balance (NEB), increased lipid mobilization, and a drop in calcium blood concentrations can emerge from increased energy and calcium requirements for colostrum and milk production, in combination with a decrease in dry matter intake (DMI) following parturition (Bell A.W., 1995; Butler W.R. et al., 1989, Goff J.P. et al., 1997, Reinhardt T.A. et al., 2011).

Thus, all these changes are increasing the risk of metabolic and inflammatory conditions, with negative effects on animal welfare, being also a significant source of production and financial losses for the dairy industry.

Generally, the occurrence of metabolic disorders is linked to feeding, dairy farm management, and also animal genetics. Imbalanced and insufficient feeding of high

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producing dairy cows during pregnancy and prepartum, is typically associated with significant metabolic changes during the transition period, making them more susceptible to develop metabolic or even infectious disorders (Bruckmaier R.M. et al., 2017). In other words, when the homeostatic processes fail to maintain normal blood calcium concentrations during early lactation, milk fever develops.

So, this metabolic condition is regarded as a gateway disease that significantly reduces the probability of full productivity in the subsequent lactation. Mild milk fever develops in the majority of cows during the peripartum period and it has been linked to certain calving difficulties, such as retained placenta, uterine prolapse, metritis, mastitis, ruminal stasis, immune system depression, and generally a reduced reproductive performance, resulting in a 3–4 year reduction in productive life (Bhanugopan M.S., et al., 2014).

If for the subclinical hypocalcaemia there are less severe changes in blood calcium levels (between 5.5 and 8.0 mg/dL), and thus, no visible symptoms (Wubishet F. et al., 2016), in clinical hypocalcaemia the initial signs include ataxia, nervousness, and hyperactivity in the animal.

Moreover, poor appetite, decreased rumen motility, low body temperature, sluggish breathing, impalpable pulse, weak but rapid heartbeats (80-100 per minute) with difficulties to be heard due to diminished capacity of muscles to contract, dilated pupils, and dry muzzle are all common symptoms of this condition (Goff J., 2008). Other signs and symptoms include a tilted head to the side, splayed out hind legs, and paresis (difficulty to rise from lying down). Finally, coma and sudden death are possible outcomes (Oetzel G., 2011, Khan A., et al., 2012).

As for its incidence in dairy cattle herds according to age and breed, milk fever tends to vary between 0 - 10%, and it may reach even 25% in some herds (DeGaris and Lean, 2008). Also, according to a meta-analysis of 135 controlled studies, performed by Lean et al. (2006), the incidence of this disease ranges from 0% to 83%, a wide range that indicates there is a big potential to affect the disease's occurrence if one understands the factors that contribute to its development.

Economic losses are significant, and include those incurred as a result of on-farm death, early culling, lower milk production, and increased veterinarian and treatment expenses (Liang et al., 2017) (figure 1).

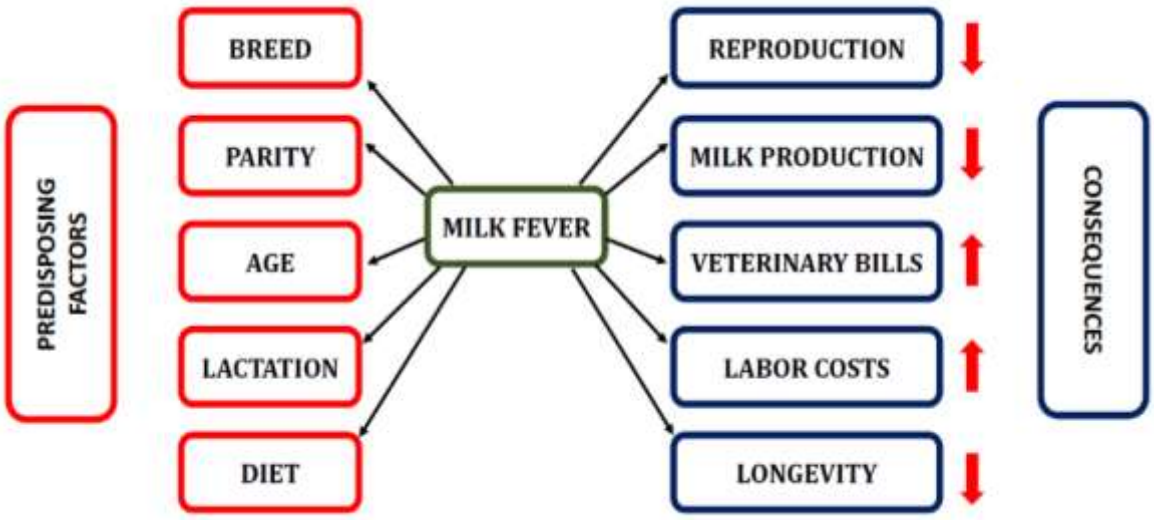

Figure 1 Predisposing factors for milk fever and its economic consequences (Dervishi E. et al., 2017)

REPRODUCTIVE CONSEQUENCES OF MILK FEVER IN DAIRY CATTLE

Dystocia is an important cause of periparturient recumbency, Chamberlain (1987) cited by DeGaris, P.J. et al. (2008), reporting that dystocia is responsible for 46% of primary recumbencies in cows, 38% for hypocalcaemia, and 16% for other causes. According to Mulligan F. et al. (2006), one may easily see the difficulties that a decreased ability of smooth and skeletal muscle contractions could cause for cows in labor.

Several scientific papers illustrate that milk fever cows are more likely to develop dystocia than normal cows. In some cases, the increased likelihood of dystocia was found to be six times compared to normal cows, while others indicated an increased probability of around 2.5 to 3 times compared to normal cows (Correa et al., 1993).

In 1984, Risco et al., stated that hypocalcaemia was found to be related to **uterine prolapse** in multiparous dairy cows and, in conjunction with other factors, is indicated as an etiologic factor for this puerperal disease in dairy cattle.

Also, within the study performed by Mulligan F. et al. (2006), 19% of cows with uterine prolapse were classified as having severe hypocalcaemia (serum calcium 4mg/dl), while another 28% were classified as having moderate hypocalcaemia (serum calcium 4.1 to 6.0mg/dl).

Later, several studies have shown that cows with subclinical hypocalcaemia are more likely to experience dystocia and uterine prolapse (Martinez N.C.A. et al., 2012). Regarding the link between milk fever and retained fetal membranes, the same author claimed that cows diagnosed with subclinical hypocalcaemia were more likely to develop **retained placenta** defined as the lack of expulsion of fetal membranes within 12 hours of parturition.

Various studies indicate an increased risk of retained fetal membranes following milk fever, milk fever cows being up to three times more likely to develop this pathological condition compared to normal cows (Houe et al., 2001). Milk fever has been reported to have a direct effect on the occurrence of retained placenta, doubling the likelihood of a retained placenta (Erb et al., 1985).

Furthermore, according to Correa M.T. et al. (1993), because milk fever is a risk factor for dystocia, which in turn is a risk factor for retained placenta, there is a significant indirect influence of milk fever on retained placenta as well.

Melendez P, et al. (2002) reported that the plasma calcium levels in cows with retained fetal membranes was considerably lower than the concentration in cows with normal placental expulsion in their study. Gild C. et al. (2015), also found that cows with subclinical hypocalcaemia experienced retained placenta in some cases. As a result, there is a clear correlation between the development of milk fever and the prevalence of retained placenta (figure 2).

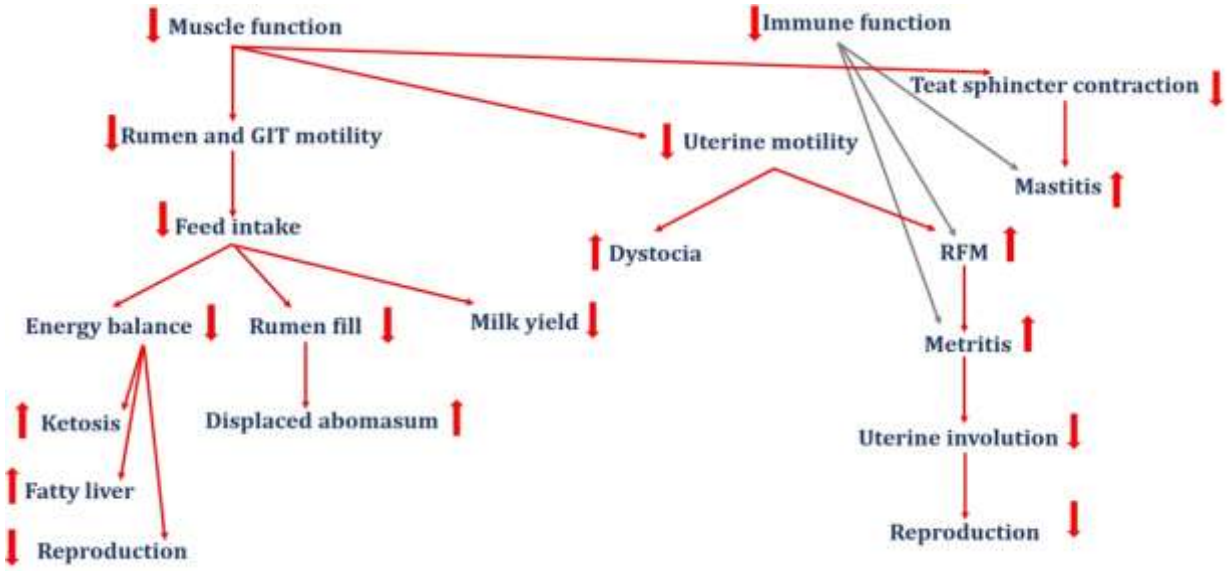

Figure 2 Consequences of milk fever and subclinical hypocalcaemia (adapted from Mulligan F et al., 2006)

METRITIS AND ENDOMETRITS

Martinez et al. (2012) conducted a prospective cohort study in which serum calcium concentrations were assessed on days 0, 1, 2, 3, 4, 7, and 12 after parturition in cows classified as metritic or healthy. Hypocalcaemia was more severe and persisted for a longer period of time in cows with metritis than in cows without this postpartum disease.

Also, according to the findings, hypocalcaemia was found to affect immunological function in this study as well. This finding was corroborated with a subsequent study conducted later (Martinez et al., 2014), which found that healthy cows with induced subclinical

hypocalcaemia had decreased appetite, impaired metabolism, and immune cell function.

Waldron et al. (2003) and Kvidera et al. (2017), on the other hand, proven that infusion of lipopolysaccharides, which induces an extensive immunological response, causes a drop in serum calcium levels. Thus, it is necessary to clarify if hypocalcaemia is a cause or a co-occurring condition of infectious diseases. According to Curtis C.R. et al (1983), and Goff J.P. et al. (1997) cited by Seifi H.A. (2017), metritis is linked to subclinical hypocalcemia.

This is possible, because metritis is more likely to occur under hypocalcemic situations, when immunological function may be compromised and muscular contraction reduced (Murray R. et al., 2008; Martinez N. et al., 2012).

Within a study on 110 dairy cattle, Martinez et al. (2012) discovered that cows who had calcium levels below 2.14 mmol/L at least once between 0 and 3 days in milk, had a 4.5-fold higher risk of developing metritis. Also, a study published by Rodríguez E.M. et al. (2017), states that multiparous cows with subclinical hypocalcemia had a 4.85 higher risk of developing metritis compared to normal-calcium animals.

The associations between milk fever, dystocia, and retained fetal membranes, along with the reported link between milk fever and periparturient immunosuppression, provide a good foundation for the postulated association between milk fever and endometritis (Kimura et al., 2006).

Compared to healthy cows, Whiteford and Sheldon (2005) discovered a higher prevalence of endometritis in cows with clinical hypocalcemia. Thus, although several publications link milk fever to complications during or around parturition, it is very likely that many farm veterinarians treat retained placenta and poor fertility issues without considering milk fever and subclinical hypocalcemia as possible contributing factors to a lower reproductive efficiency and higher culling rates.

In cows after parturition, uterine contamination is inherent, although it is progressively removed by uterine involution, discharge of lochia, and mobilization of immunological defenses (Bretzlaff 1987).

However, uterine bacterial contamination persists more than three weeks in 40% of cows, and nearly half of these animals develop clinical endometritis (Sheldon I.M. et al., 2004).

Hypocalcemia affects smooth muscle contraction (Goff J.P., 2008) and has been linked to a decreased contractility of the myometrium. Between 15 and 32 days postpartum, cows having a history of milk fever in the same lactation have larger uterine horn diameters (Goff J.P., 2014).

FERTILITY

Cows who have recovered from clinical hypocalcaemia require more services per conception and have prolonged intervals between calving and conception after they have recovered (Borsberry and Dobson 1989). However, it is unclear if clinical hypocalcaemia, which occurs during the first few days after parturition, has an impact on reproductive function several weeks after the parturition.

When it comes to optimal fertility, hypocalcaemia can interfere with one or more of the three main events of the postpartum period that must occur in order to achieve it: the restoration of normal ovarian cycle activity, uterine involution, and the reduction of uterine bacterial contamination (Sheldon I.M. et al., 2004).

Clinically or subclinically hypocalcemic animals have a prolonged interval between calving and their first postpartum ovulation (Risco et al., 1994).

Additionally, subclinical hypocalcaemia results in smaller follicles at first ovulation in cows (Kamgarpour and others 1999) this observation being significant, as cows with follicles larger than 8 mm in diameter between 14 and 28 days postpartum have shorter calving to conception intervals (Sheldon I.M. et al., 2004).

Given the fact that uterine involution and lochia discharge are dependent on rhythmical uterine contractions, which are suppressed by hypocalcaemia, it is not surprising that clinical hypocalcaemia is related to delayed uterine and cervical involution (Risco et al., 1994).

PREVENTION

Preventing milk fever is economically beneficial for dairy farmers due to the reduced production loss, death loss, and veterinary expenses associated with milk fever. Numerous nutritional management measures have been used to control hypocalcaemia and mobilize calcium in dairy cattle, including the use of anionic salts, low calcium ion diets, and vitamin D supplementation (Amaral-Phillips D., 2017).

Before parturition, low calcium diets improve the release of Parathyroid Hormone. This stimulates osteoclasts in the bone, promotes calcium resorption in the bone, stimulates renal tubules to resorb urine calcium and start making 1,25-dihydroxyvitamin D. As

a result, when lactation begins, the calcium homeostatic pathways become active and capable of preventing hypocalcaemia (Oetzel G.R., 2011).

According TO Jesse P.G. et al., (2018) feeding cows with **calcium poor diets** during the dry period is one of the methods to prevent milk fever.

This can be achieved by providing less than 50 g/ each day. As a result, calcium-rich forages such as alfalfa should be removed from the animal's diet. Corn silage and grass hay should be fed often throughout the dry period to help lower calcium levels (Angassa T., 2019).

Bhanugopan M.S. and Lievaart J., (2014) found that all farmers used hay, straw, and grain as a general nutrition plan throughout the dry period. Practically, grain feeding helps the rumen adapt quickly to the high-energy diets given post-partum, and grains also have a low calcium content.

Dietary cation-anion balancing (DCAB) represents a nutritional strategy to prevent milk fever in early lactation, but also to enhance the cow's health and performance (Patel V.R. et al., 2011).

It is a typical prophylactic method that involves providing anionic salts to decrease the cation anion difference in the diet and has been successfully adopted in the dairy farms (Martín-Tereso J. et al., 2014). The objective of this form of supplementation is to decrease the amount of absorbable cations like sodium and potassium in the diet and increasing the amount of accessible anions such as chlorine and sulfur monoxide (Goff J., 2008).

Given the increased potassium content of dry fodder, is recommended to avoid feeding cows with excessive amounts of dry fodder, in order to prevent milk fever, being essential to include silage and succulent / green fodder in a significant percentage of the dry cow's diet, since they contain less potassium (Thirunavukkarasu M. et al., 2010).

Bhanugopan M.S. et al. (2014), stated that one method of preventing milk fever in cows, is to administer orally supplements with **calcium around parturition.** Later, Amanlou H. et al. (2016), indicated that 2 subcutaneous calcium infusions in the first 18 hours postpartum are correlated with a lower risk of developing postpartum disorders (metritis, clinical and subclinical endometritis and hypocalcemia) in cows from experimental group, compared to control group animals.

Magnesium is an important component of calcium metabolism, serving as a critical element of calcium metabolism in the resorption of calcium from bone by Parathyroid hormone. As Jesse P.G. et al. mentioned in a study published in 2018, magnesium supplementation is critical for preventing milk fever.

Increased magnesium supplementation was determined to be the most effective strategy for preventing milk fever (Lean I.J. et al., 2006).

In addition to the methods mentioned above, Bhanugopan M.S. and Lievaart J., (2014) recommended **vitamin D supplementation** in prepartum dry cows. This method needs injecting or feeding up to 10 million IU of vitamin D daily for 10-14 days before parturition, improving thus intestinal calcium absorption.

Regarding **body condition score (BCS)**, Ostergaard et al. (2003) and Finbar M. et al. (2006), stated that over-conditioned dairy cows during calving are up to four times more likely to develop milk fever, because they have a higher calcium output in milk. Compared to thinner dairy cattle, over-conditioned cows have a lower feed intake in the last week or ten days before parturition. This may result in a reduction in their calcium and magnesium intake to levels that predispose them to the development of hypocalcaemia.

It is essential to keep the dry cows from becoming too fat. Cows who have seen significant body condition loss during the dry period are also at susceptible to develop milk fever (Etagegnehu B. et al., 2020).

CONCLUSIONS

Taking into consideration the above mentioned, preventing milk fever is critical for mitigating the economic effect of the diseases. Nutritional techniques and body condition control are crucial for disease prevention.

According to Etagegnehu B., et al. (2020), training dairy farmers is essential for educating them about milk fever and the right composition of rations for their dairy cows.

Also, is recommended that dairy farm operators should lower the energy source of feed, particularly concentrate during calving. Farmers should be educated about the importance of observing their dairy cows 48-72 hours before and after calving for signs of milk fever.

As a final mention, additional research on the epidemiology and economic impact of milk fever in the dairy industry should be done.

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REVIEW: THE EPIDEMIOLOGY AND ZOONOTIC RISKS OF CORONAVIROSES

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Abstract

The following paper includes a synthesis of bibliographical information regarding the main diseases caused by coronaviruses, both in animals (companion and domestic) as well as in humans, observing the imprevisible tendencies of this viral family. These data are completed by an epidemiological analysis of the main events caused by coronaviruses in humans, using the available databases.
Coronaviruses are worldspread entities, producing, in humans and animals, the most diverse diseases, with digestive, respiratory or nervous symptoms in animals, some forms being very serious and with special economic implications and others mild or even clinically unexpressed; in humans, the symptoms are predominantly respiratory, in some cases beginning with digestive signs and the complications that occur may be neurological in nature. Over the years, especially since 2002 (SARS-CoV), continuing with 2012 (MERS) and more recently, from 2019, culminating with SARS-CoV-2, it has been possible to identify the trend of zoonotic transmission (from animal to human), with a particularly high pathogenic potential of these viruses, which have different rates of morbidity and mortality. Another interesting aspect is the fact that cases of anthropozoonotic transmission (from human to animal) have been reported, in the case of pets, but also of fur animals (minks).

Keywords: coronavirus; infectious disease; animals; zoonosis; pandemic.

INTRODUCTION

The first coronavirus, the avian infectious bronchitis virus, was isolated from chicken eggs in 1937. The transmissible swine gastroenteritis virus and the mouse hepatitis virus were subsequently identified from swine and mouse samples in the 1940s. In the following decades, while human coronaviruses were discovered in the 1960s, other animal coronaviruses, such as porcine hemagglutinating encephalomyelitis virus (1962), feline coronavirus (1970), canine coronavirus (1971), bovine coronavirus (1973), turkey coronavirus (1973), porcine epidemic diarrhea virus (1978) and porcine respiratory complex virus (1984) were also discovered. To study coronaviruses, various reverse genetics systems have been established and implemented since 1992 to understand viral replication, elucidate virus-host interaction, and the pathogenesis process, so that the discovery of new coronavirus vaccines is facilitated [1]. Thanks to the next generation high throughput sequencing technology, which was discovered in 2005, its application in virology paved the way for a new era of coronavirus discovery. Thus, several emerging coronaviruses, such as swine deltacoronavirus, have been identified (2009) and characterized (2014) [2].

In some circumstances, coronaviruses can be transmitted from animals to humans, adapt to the human species and then spread to the human population without the subsequent involvement of animals. Therefore, in humans, infections with the following coronaviruses have been described: Severe acute respiratory syndrome coronavirus, Severe acute respiratory syndrome coronavirus type 2, Middle East respiratory syndrome coronavirus, and Human coronavirus -229E, -NL63, -OC43 and -HKU1. The most recent human Coronavirus infection is COVID-19, produced by SARSCoV-2, declared a pandemic by the World Health Organization on March the 11th, 2020. In animals, the following coronaviruses are more important: Bovine coronavirus (BCV), Equine coronavirus (ECoV), Transmissible gastroenteritis coronavirus of pigs (TGEV), Porcine respiratory coronavirus (PRCV), Porcine epidemic diarrhea virus (PEDV), Porcine hemagglutinating encephalomyelitis virus (PHEV), porcine acute diarrhea syndrome virus (SADS-CoV), Porcine deltacoronavirus (PDCoV), Canine enteric coronavirus (CCoV), Canine respiratory coronavirus (CRCoV), Feline

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coronavirus (FCoV), Infectious bronchitis virus (IBV) and Turkey coronavirus (TCoV) [3].

The world is currently struggling to mitigate the consequences for public health and to survive the socio-economic crisis caused by measures taken around the world to prevent the spread of COVID-19. In addition, SARS-CoV-2 demonstrates the ability to infect and sometimes cause respiratory damage in many species of mammals. Transmission from humans to dogs, cats, lions, tigers and minks took place and, in the latter case, intraspecific transmission from one mink to another has been observed and notified by the OIE in many countries. The involvement of different species of mammals, which are domestic animals, but also wild animals, in the circulation of SARS-CoV-2 indicates the need for surveillance, intervention and management strategies such as "One Health" to mitigate the effects of this potential panzootic virus [4, 5, 6].

RESULTS

1. Animal coronaviruses

According to current taxonomy, coronaviruses are classified as one of two genera of the Coronavirinae subfamily, the Coronaviridae family of the Nidovirales order. Coronaviruses (CoV) are divided into four genera based on phylogenetic links and genomic structures: Alpha-, Beta-, Gamma- and Delta-CoV. Almost all alpha-CoV and beta-Cov have mammalian hosts, while gamma-CoV and delta-CoV are commonly found in avian hosts, although some of them can also infect mammals. Members of this large family are considered to be the causative agents of respiratory, enteric, hepatic and neurological diseases in birds and mammals [3].

1.1.Avian coronaviruses

Avian coronaviruses belong to the Gammacoronavirus genus which includes three major species: infectious bronchitis virus (IBV), pheasant coronavirus (PhCoV) and turkey coronavirus (TCoV). IBV or IBV-like gammacoronaviruses have been found in other bird species, such as peacocks, partridges, pigeons, guinea fowl and various species of wild birds [7]. Infectious bronchitis virus, the first coronavirus discovered, is by far the most important and best studied gammacoronavirus and is therefore considered the prototype of the genus. This virus is of great economic importance to the poultry industry around the world, affecting both the performance of farmed poultry for meat and eggs. IBV is an extremely contagious disease that affects the respiratory, reproductive and renal systems, with a different degree of severity depending on the viral strain involved [8].

1.2.Domestic carnivores coronaviruses

In dogs, two types of coronaviruses are known: two alpha-coronaviruses, namely CCoV-I and CCoV-II, and betacoronavirus CRCoV. CCoVs are gastrointestinal viruses with fecal-oral transmission, frequently isolated in dogs around the world, but in most cases, they are able to cause a very mild gastrointestinal disease in puppies or are completely asymptomatic [9].

However, a recently characterized strain (CB/05) has led to a fatal evolution due to the systemic spread of the virus [10]. Moreover, CCoV-infected intestinal villi appear to increase the susceptibility of cells to canine parvovirus (CPV) infection. This leads to a synergistic action that ends with a much more serious disease than the one that both viruses can cause separately [11].

Unlike CCoV, CRCoV beta-coronavirus, also known as group II canine coronavirus, causes mild respiratory signs in dogs and is considered the etiological agent of canine infectious respiratory disease (CIRD) along with other viral and bacterial agents. This beta-CoV is genetically related to one of the human coronaviruses responsible for the common cold, namely HCov-OC43, and the bovine coronavirus BCoV [12, 13].

FCoV type I and FCoV type II feline coronaviruses belong to the genus Alphacoronavirus. Both genotypes cause a mild enteric disease that, in most infected cats, shows no signs of disease. However, enteric coronaviruses can undergo a mutation in the host and acquire the ability to infect monocytes and macrophages, causing systemic disease. In this form, called feline infectious peritonitis (FIP), the virus causes a serious disease related to an intense immune response, with a fatal result in most cases [14].

1.3.Porcine coronaviruses

Six coronaviruses can cause infections in pigs. These include four alfacoronaviruses, namely porcine transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCoV), porcine epidemic diarrhea virus (PEDV) and SADS-CoV, a betacoronavirus, namely porcine viral hemagglutinitis virus, and porcine deltacoronavirus (PDCoV). TGEV, PEDV, SADS-CoV and PDCoV are responsible for acute gastroenteritis in pigs. PRCoV causes a mild respiratory disease, and PHEV is the causative agent of neurological and/or digestive diseases in pigs [15].

1.4.Bovine coronaviruses

The most common bovine coronavirus is BCoV. This virus is capable of causing many clinical forms, including severe enteric disease in

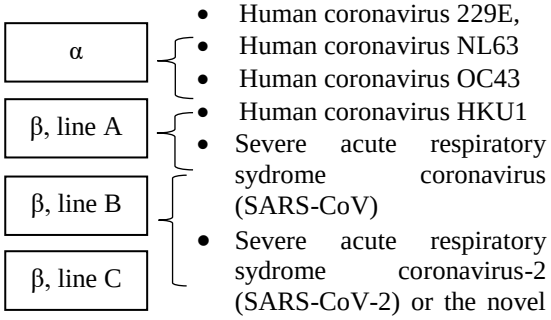
newborn calves, disease with severe enteric evolution in adult dairy cattle, and respiratory disease in cattle of all ages [16]. Interestingly, HCoV-OC43 probably evolved from ancestral BCoV strains that crossed the interspecies barrier and established an infection in humans around 1890, following a 290-length nucleotide deletion downstream of the spike gene [17].

1.5.Equine coronaviruses

The only CoV that has been discovered so far in horses is ECoV, which belongs to the Betacoronavirus genus. ECoV is a newly recognized enteric virus of adult horses that has been associated with fever, lethargy and anorexia, as well as colic and diarrhea. Outbreaks have been reported in Japan, Europe and the USA since 2010 [18].

2.Human coronaviruses

Human coronaviruses fall into the first two categories of the most widely used classification, namely α-coronaviruses (Alphacoronavirus) and β-coronaviruses (Betacoronavirus).



Hosts, reservoirs and infected animals: Bats are the carriers of most viral genotypes. [19]

2.1.Human alfacoronaviruses (HCoV-229E și HCoV-NL63)

These cause acute rhinitis, acute pharyngitis, acute laryngitis, rarely bronchiolitis, acute bronchitis, viral pneumonia. Gastrointestinal symptoms (nausea, diarrhea, vomiting) were encountered, but it was not possible to specify exactly whether these clinical signs were caused by the virus itself, or by another opportunistic pathogen. Severe manifestations occur only in immunosuppressed patients. However, most of the time, the disease goes unnoticed because it has no clinically obvious manifestations [20, 21].

History: The first human coronaviruses were discovered in the 1960s (the first The viral strain was named B814, however, because very few cases were reported at the time and some of them were found to be actually HCoV-229E, it was concluded that B814 strain is actually HCoV-229E) by researchers at the Common Cold Unit in Salisbury, UK, who showed that common colds can be caused not only by rhinoviruses but also by a coronavirus. In 2003, in a laboratory in the Netherlands, a new coronavirus was discovered, namely HCoV-NL63, a virus that is second to the global spread, being one of the most common viruses. It is considered that some animals, such as bats, are a real reservoir of these viruses, the disease being considered a zoonosis. A new alpaca virus, ACoV, was discovered in 2007, which in 2012 was shown to be 92.2% identical to HCoV-229E after sequencing the viral genome [22, 23, 24, 25, 26].

Epidemiology: Transmission is aerogenic and by direct contact and rarely indirectly, through objects newly contaminated with virus (handkerchiefs). They are ubiquitous viruses and have the phenomenon of "seasonal viruses", being more common at certain times of the year.

Clinical signs: Unpleasant sensation of stuffy and dry nose, followed by repeated sneezing and profuse rhinorrhea (initially watery, then yellowish). Tickling and burning sensation of the nasal mucosa. Smell, taste and appetite are diminished, and digestive signs such as diarrhea, nausea may occur [9]. A hoarse, dry, irritating cough also occurs frequently. fever rarely exceeds 38.2°C in the first 2-3 days. After 5-7 days, all symptoms gradually go away. Sometimes, in more severe cases, nervous signs may appear [20, 27, 28, 29].

Prophylaxis and control: There is no specific vaccine yet. Routine prophylactic measures (isolation, disinfection). A good measure of hygiene is to use cellulose handkerchiefs, then throw them in special closed boxes or on fire [30].

2.2. Human betacoronaviruses

2.2.1. Line A Betacoronaviruses
HCoV-OC43

Discovered in 1967 in a laboratory in Maryland, USA, this virus is the most common strain of coronavirus worldwide and is associated with some of the most severe pathological manifestations of HCoV. In 2005, following the sequencing of its entire genome, a link was discovered between OC43 and BCoV (of bovine origin), which proves its zoonotic origin, finding a link with the viral epidemic of 1890, when "Russian/Asian Fever" killed 1 million people [29, 31].

HCoV-HKU1

Discovered in 2005 in a laboratory in Hong Kong, China, this virus is associated with seizures caused by febrile conditions, predominantly among children. Line A betacoronaviruses are found worldwide and have the phenomenon of "seasonal viruses", being more common at certain times of the year.

Epidemiology: Transmission is aerogenous and by direct contact and rarely indirectly, through objects newly contaminated with virus (handkerchiefs). They are ubiquitous viruses.

Clinical signs: Unpleasant sensation of stuffy and dry nose, followed by repeated sneezing and profuse rhinorrhea (initially watery, then yellowish). Tickling and burning sensation of the nasal mucosa. Smell, taste and appetite are diminished. A hoarse, dry, irritating cough also occurs frequently. fever rarely exceeds 38.2 ° C in the first 2-3 days. After 5-7 days, all symptoms gradually go away.

Prophylaxis and control: There is no vaccine yet. Routine prophylactic measures (isolation, disinfection). A good measure of hygiene is to use cellulose handkerchiefs, then throw them in special closed boxes or on fire [28].

2.2.2. Line B Betacoronaviruses

SARS-CoV

(Severe Acute Respiratory Sydrome - Coronavirus)

History: The first case was reported on November the 16th, 2002 in China, in Guandong Province. In the following months, the disease was reported in more than 8,000 people (including 1,706 medical staff). Of these cases, 774 died, the lethality reaching 9.6%. The epidemic has spread to 29 countries on 5 continents **(Figure 1)**. No other cases were reported after this epidemic in 2002-2003, the last case being treated in July 2003. The disease was considered to be an extremely fatal infection.

Epidemiology: The natural reservoir is probably bats, from which it is believed that the virus reached civets, then humans, and also, patient 0. The general source of infection is the human with symptomatic infection, a sick person being able to infect an average of up to 3 other people (*Basic reproduction number*). The disease is transmitted directly through nasopharyngeal droplets in the air and indirectly from infected people, through direct contact with them or through objects contaminated with infectious nasopharyngeal secretions [32].

Clinical signs: Severe acute respiratory syndrome: fever (>38°C), chills, myalgia, dry cough, dyspnea and severe impairment of respiratory function (respiratory distress) [33].

Prophylaxis and control: There is no specific or preventive antiviral treatment (vaccine), the treatment being only symptomatic. In the absence of a vaccine, the prevention of SARS transmission involves: epidemiological triage, early detection and isolation of cases, surveillance and quarantine of contacts, sanitary border surveillance [34].

SARS-CoV-2

(Severe Acute Respiratory Syndrome-Coronavirus 2 (SARS-CoV-2) or the novel coronavirus 2019 (2019-nCoV)

History: The first case of nCoV was detected on November the 17th, 2019 in Wuhan, China, from where it spread to most Chinese provinces and several countries around the world, including Europe.

Epidemiology: The primary source of infection is not yet known (probably contact with bat-infected animals; it was previously assumed that the source of infection could be snakes). Transmission is thought to be similar to that of respiratory MERS-CoV and SARS through nasopharyngeal secretions (Flügge drops), produced when an infected person coughs, sneezes or speaks and through direct or indirect contact with nasopharyngeal secretions from an infected person laid on their hands or surfaces (tables, desks, clothing, etc.) in case they touch their eyes, nose or mouth. Initially, a sick person can infect on average up to 3 other people (*Basic reproduction number*), but, against the background of the widespread transmission among the human population, more aggressive variants have appeared and with a higher degree of contagion. Transmission is also possible when there is direct contact with fluids (blood, feces, urine, saliva, semen) from an infected person. The virus is easily transmitted from one person to another. The disease occurs in people who have had close contact with a confirmed or probable case of COVID-19 or have had a history of travel to the affected areas (China, Italy, France, etc.) within 14 days prior to the onset of symptoms. The degree of danger is represented by the carrying of the virus by asymptomatic people, who can spread the virus if they do not respect the imposed hygiene norms, infecting the people around [35, 36].

SARS-CoV-2 uses surface-localized glycoprotein (S) to interact with cells that express the angiotensin 2 conversion enzyme receptor (ACE2) [19]. Glycoprotein S comprises two distinct functional subunits, namely S1 and S2, which are involved in receptor recognition and membrane fusion, respectively [20]. The

interaction between the ACE2 protein and SARS-CoV-2 S involves a C-terminal domain of the S1 subunit, also called the receptor binding domain (RBD), which is a determinant of viral infectivity and host specificity [37]. The sensitivity of different animal species to SARS-CoV-2 is worrying given its potential for transmission, and its understanding is crucial for controlling the spread of the virus. Indeed, coronaviruses have a strong potential for cross-transmission, caused by their ability to genetically adapt **(Figure 2)**, especially when they involve RBD [38].

The range of hosts for SARS-CoV-2 can be extremely wide due to ACE2 expression in a wide spectrum of vertebrates. However, the variation of ACE2 residues involved in the RBD-receptor interaction may influence the susceptibility of different species to this CoV. Thus, a comparative analysis of ACE2 protein sequences can be used to predict their affinity for SARS-CoV-2 S-glycoprotein binding and, consequently, the species that can serve as a host for this virus [19].

Li et al. [37] first aligned the amino acid sequence of ACE2 from different species, including humans, five non-human primates, eight domestic animals (cats, dogs, cattle, sheep, goats, pigs, horses and chickens), three wild animals (ferret, civet and Chinese bat) and two species of rodents (mice and rats). The authors found that human and non-human primates share identical sequences in some regional residues. The high degree of sequence similarity observed in most domestic and wild animals implies that ACE2 from these animals can recognize SARS-CoV-2. Thus, these animals may be susceptible to infection. On the other hand, rodents and chickens probably cannot meet the conditions of susceptible hosts.

More recently, Damas et al. [38] used comparative genomic combinations and structural analyzes of proteins to study the conservation of ACE2 in 410 vertebrate species and its potential to be used as a SARS-CoV-2 receptor. This study confirmed the high susceptibility of primates to SARS-CoV-2 infection. On the other hand, this study predicted a wider group of species that could serve as a reservoir or intermediate host for this virus, with mammals having a medium to high score in terms of their tendency to ACE2 to bind to SARS-CoV-2 protein S.

Based on the results of molecular studies, the ACE2 proteins of non-human primates and most domestic and wild animals closely resemble the human ACE2 receptor. However, the results may differ between susceptibility to natural infection and that observed experimentally. Indeed, these studies are based exclusively on in silico analyzes and focus on a small number of amino acid residues, i.e. 25 amino acids corresponding to known SARS-CoV-2 S protein binding sites [38]. Cross-species transmission is based not only on the presence of the receptor, but also on the levels of ACE2 expression in the respiratory mucosa and on the presence of other cellular factors necessary for viral replication. Therefore, these studies need validation by experimental infection on animal models or examples of natural infections.

- ***The zoonotic and anthropozoonic character of SARS-CoV-2***

The current hypothesis that the primary source of infection was a probable contact with infected animals has not yet been confirmed. It is expected the connection between bats and pangolins, which are a genus of tropical mammals of the order of edentates whose body and tail are covered with horn-like scales, which feed on insects; it was previously thought that the source of the infection could be snakes. In any case, it is considered to be a zoonosis. Unfortunately, there are no clear data on the precise origin of this pandemic, and speculation can still be easily elaborated [35, 39].

One noteworthy aspect is the fact that there have been numerous situations in which pets, in particular ferrets and cats, have shown a susceptibility to this virus. Dogs and hamsters were also susceptible, but to a much lesser extent. In most cases, the pets took the virus from the owners who tested positive for COVID, the exposure being long, the owners being quarantined at home with them. The animals usually showed no clinical signs, only through molecular (RT-PCR) and serological tests (especially ELISA) were found, either traces of virus in the animals' blood or anti-SARS-CoV-2 antibodies. Most cases were found in outbreak areas, especially in China and Italy [40,41].

Another situation that should not be overlooked is the situation of outbreaks of SARS-CoV-2 in mink fur farms. More than 50 million minks are raised annually for fur, mainly in China, Denmark, the Netherlands and Poland. Outbreaks have occurred in the Netherlands, Denmark, Spain, Sweden, Italy and the United States, killing millions of animals [42, 43, 44, 45].

A special phenomenon took place on farms in the Netherlands. In 4 of the 16 farms studied (in the SE part of the Netherlands, in April and May, 2020), minks are thought to have become ill from workers, and the virus was transmitted rapidly from animal to animal (minks have had fairly severe respiratory signs, but there were also asymptomatic animals) then other workers, who were healthy at the time, took the virus from minks. Finally, 66/97 (67%) of farm staff tested

positive for SARS-CoV-2 through PCR/or serologically. The same viral strain was isolated from minks and humans. A favorable aspect is that the possible exposure to the virus in the areas adjacent to the farms proved to be negligible, the infected people being represented only by the workers who were repeatedly exposed [43]. The total cases and outbreaks of SARS-CoV-2 recorded in animals can be seen in **Table no. 1**, and the outbreaks worldwide can be seen in **Figure no. 3**.

2.2.3.Line C Betacoronavirus

MERS-CoV

(Middle East Respiratory Syndrome - Coronavirus)

History: The first cases of MERS-CoV virus infections occurred in June 2012 in Saudi Arabia and Qatar. In May 2015, 1180 cases were confirmed and 483 (41%) deaths were reported. The countries of the Middle East - Saudi Arabia, Qatar, Jordan and the United Arab Emirates - were predominantly affected, but cases imported into the United Kingdom, the Netherlands, the USA and Asia were reported. The largest outbreak outside the Middle East occurred in South Korea in 2015, affecting several hospitals and 185 people, causing 36 (19.5%) deaths. From 2012 to January 2020, 2,519 cases of MERS-CoV virus infection and 866 deaths were reported to the World Health Organization (WHO), so we face a rate of approximately 34.3% among fatal cases (**Figure no. 4**).

Epidemiology: The natural reservoir is probably the bats that transmit the virus to the intermediate host - camelids, especially dromedaries, which are the source of infection for humans, the infection being settled after consuming milk or camel meat. Most cases of MERS occurred in health care facilities. The virus is not easily transmitted from one person to another except in close contact, as in the case of caring for a patient without the application of protective measures. Cases of infected but asymptomatic people have also been reported. Transmission between individuals in the general population is limited; a sick person can infect on average up to 0.3-0.8 other people (*Basic reproduction number*), this number <1 is encouraging, as it indicates that although the disease still exists worldwide (it has not been completely eradicated), affects an extremely limited number of people, and its completely descending slope highlights the end character of the disease, the virus will soon disappear on its own [46, 47, 40].

Clinical signs: The symptoms are extremely similar to SARS-CoV- 2; these include: fever, dyspnea and cough. Gastrointestinal symptoms with diarrhea, vomiting, and abdominal pain may also be present. Unusual compared to other coronaviruses is the presence of acute kidney problems. Most (90%) develop severe pneumonia or respiratory distress syndrome and require intensive care [46, 47, 48, 40].

Prophylaxis and control: There is no specific or preventive antiviral treatment (vaccine), the treatment being only symptomatic. Washing hands with soap and water, or other disinfectants, especially after coughing, sneezing. Covering the nose and mouth with a paper handkerchief in case of coughing or sneezing and then throwing the handkerchief in the trash for safe disposal. Avoiding touching the eyes, nose and mouth with hands, as this can transmit the virus after contact with contaminated surfaces. Avoiding contact with infected people and applying facial masks in overcrowded places. Avoiding close contact with sick people, such as kissing, exchanging drinks and eating utensils. Frequent cleaning and disinfection of affected surfaces, e.g. of toys and door handle [40].

DISCUSSION

SARS-CoV-2 can infect and sometimes cause disease in many species of animals. Of all, the closest phylogenetically to humans (macaque, orangutan and chimpanzee) and possessing a virus receptor identical to ours are certainly the most susceptible to infections and disease development. Also, cats have certainly been shown in both laboratory studies and reported cases to be susceptible to the virus and sometimes able to transmit it to other animals. Frequently, the virus transmitted between cats has caused visible symptoms, which are mild in most cases (sneezing, loss of appetite, apathy, weakness, and sometimes lacrimal hypersecretion have been observed), while in few cases severe respiratory symptoms have been reported with bilateral pneumonia and shortness of breath [49].

Dogs are the only species of Canidae that has been reported to contact the SARS-CoV-2 infection and, in most cases, no signs of disease have been observed in the affected animals. Only in the USA, passive surveillance has identified nonspecific symptoms in older animals with various comorbidities that do not allow the understanding of the pathogenicity mechanism in dogs. In all documented cases where the virus infected domestic animals, transmission occurred from human to animal. Therefore, pets do not pose a risk to the transmission or maintenance of

the virus in the human population and it is mandatory to avoid what happened in China, where many pets were killed or abandoned, following the public statement of a member of the the team of senior experts from China's National Health Commission that pet owners should take extra care of their animals [50].

The situation of mustelids requires a different approach. As observed in experimental infection studies, ferrets, once infected with the virus, develop signs of respiratory disease and transmit the virus to other ferrets [49]. Mink farms have been affected in almost all European countries, but also in the USA. In the cases reported by the Dutch authorities, where strict veterinary surveillance was implemented, in 60% of cases, the positivity was observed post-mortem, with no signs of the disease observed in the farm, while in the remaining 40%, the cases were identified after signs of disease in the farm (increased mortality, loss of appetite, etc.). In addition, serological investigation detected up to 70% of animals with antibodies [51].

As described in Spain, the first weak positivity, defined as inconclusive, was observed in the second half of May. The virus circulated on the farm for at least 6 weeks, until July, when the epidemic broke out on the farms, reaching a positive rate of 86.7%. In Italy, a lower spread of the virus was observed in the monitored farms, with only a few samples that gave positive results [52].

CONCLUSIONS

The spread of coronavirus between animals inevitably leads to mutations of the virus. The spike protein of the virus is the most affected by these mutations. The spread of SARS-CoV-2 in mink farms in Denmark has led to the emergence of a new variant of the virus called Y435F, which has also been observed in human populations living in the district. However, the Y435F mutation has already been observed in other countries (Russian Federation, South Africa, Switzerland and the United States). Thus, it can be stated that recombination within viral structural proteins between coronaviruses from different hosts may be responsible for cross-transmission. These characteristics could allow the virus to become endemic in some populations of domestic and wild animals.

Individuals working in contact with animals must be provided with appropriate safety equipment, thus reducing the viral spread from human to animal. Also, worker testing, contact tracking, isolation and quarantine should be initiated immediately when a human case is related to an animal farm, and this approach should not be limited to highly sensitive farm animals such as minks. Hunting and wildlife farming bring people into contact with wildlife, and these animals often suffer from debilitating and immunosuppressive disorders that encourage zoonotic outbreaks. Therefore, veterinary surveillance of zoonotic diseases should be maintained and implemented where it is not present, in order to detect early the presence of SARS-CoV-2 in animals. Isolated strains of SARS-CoV-2 should be sequenced, and these sequences obtained from infected animals should be made available to the scientific community to monitor the occurrence or spread of virus variants adapted to animals, but still potentially dangerous to humans. Given the availability of vaccination in the human population, veterinary surveillance should be strengthened among susceptible species, but also other animal species, including wild animals, with a particular focus on wild mustelids.

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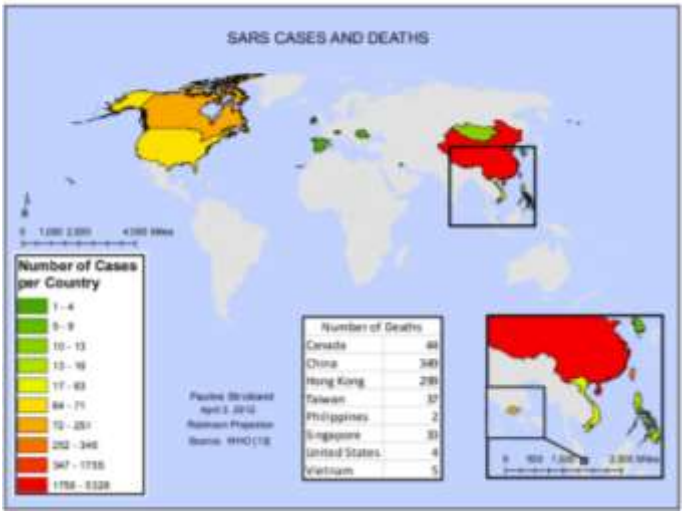
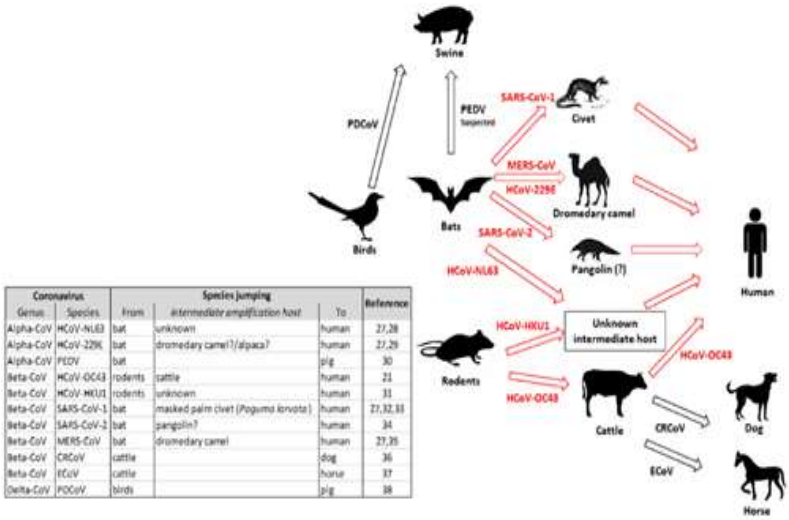


Fig. 1. : SARS-CoV: Number of cases and deaths

Source: https://upload.wikimedia.org/wikipedia/commons/3/33/Sars_Cases_and_Deaths.pdf



Coronavirus		Species jumping			Reference
Genus	Species	From	Intermediate amplification host	To	
Alpha-CoV	HCoV-NL63	bat	unknown	human	27,28
Alpha-CoV	HCoV-229L	bat	dromedary camel?/alpaca?	human	27,29
Alpha-CoV	PEDV	bat		pig	30
Beta-CoV	HCoV-OC43	rodents	cattle	human	21
Beta-CoV	HCoV-HKU1	rodents	unknown	human	31
Beta-CoV	SARS-CoV-1	bat	masked palm civet (Paguma larvata)	human	27,32,33
Beta-CoV	SARS-CoV-2	bat	pangolin?	human	34
Beta-CoV	MERS-CoV	bat	dromedary camel	human	27,35
Beta-CoV	CRCoV	cattle		dog	36
Beta-CoV	ECoV	cattle		horse	37
Delta-CoV	PDCoV	birds		pig	38

Fig. 2 - Representation of interspecies transmission of coronaviruses. Red arrows indicate cross-transmission involving an intermediate amplifier host (diagnosed, suspected or undetermined). Black arrows indicate a direct transmission between two species [6]

Table no. 1 : Total cases and outbreaks of SARS-CoV-2 in animals

Continent	Species	Outbreaks	Cases	Slaughtered	Deaths
Africa	Lion	1	3	0	0
	Puma	1	1	0	0
The Americas	American Mink	18	4	0	14130
	Lion	10	10	0	0
	Tigers	15	23	0	0
	Gorilla	4	16	0	0
	Dog	106	87	1	5
	Asian Otter	4	11	0	0
	Mustelidae	10	263	10.917	248
	Amur Leopard	2	0	0	0
	Snow Leopard	3	5	0	0
	Cat	94	89	0	9
	White-tailed deer	1	8	0	0
	Puma	3	2	0	0
	Jaguar	2	0	0	0
	Rabbit	1	0	0	0
	Pig	1	0	0	0

Asia	Tiger	3	6	0	0
	Dog	22	21	0	0
	Cat	11	14	0	0
Europe	Mustelidae	43	22.729	0	1.578
	Dog	7	8	0	0
	Cat	12	13	0	0
	American Mink	6	338	0	338
	Ferret	1	1	0	0
	TOTAL	**381**	**23.652**	**10.918**	**16.308**

Fig. 3.: Outbreaks of SARS-CoV-2 in animals worldwide
Source: https://www.oie.int/en/scientific-expertise/specific-information-and-recommendations/questions-and-answers-on-2019novel-coronavirus/events-in-animals/

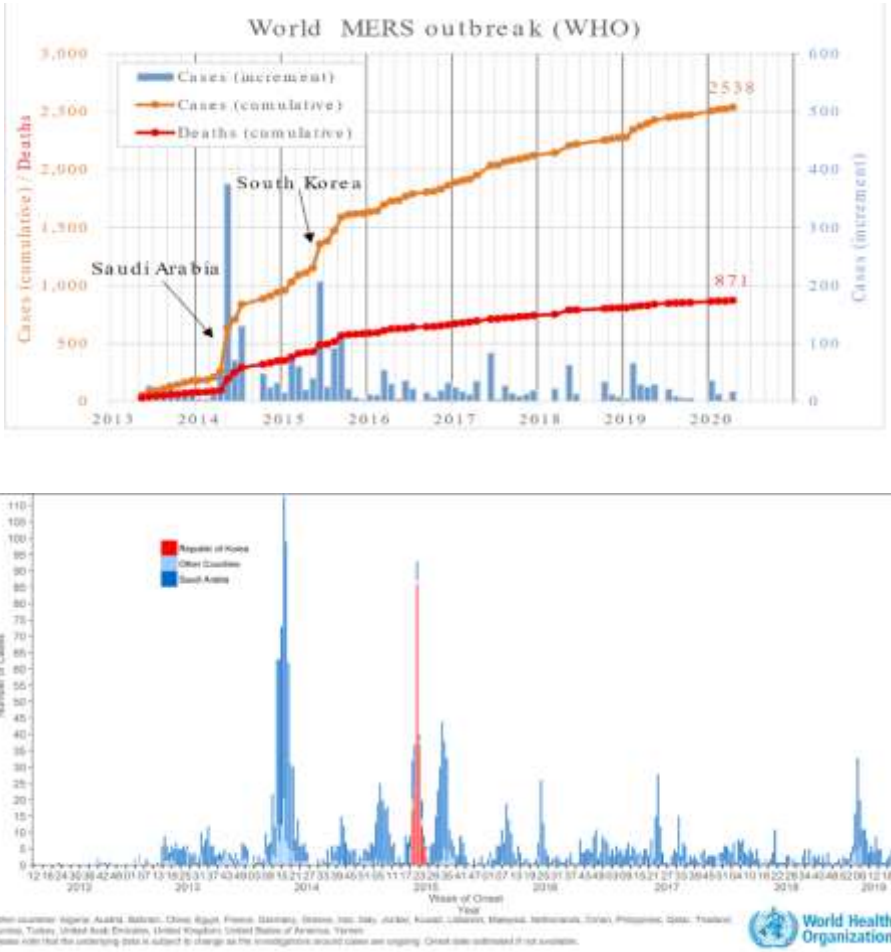


Fig. 4: MERS: Number of cases and deaths reported worldwide in 2012-2020
Source: https://www.who.int/health-topics/middle-east-respiratory-syndrome-coronavirus-mers#tab=tab_1

A REVIEW: BOVINE SPONGIFORM ENCEPHALOPATHY ASSOCIATED WITH PRNP GENE POLYMORPHISMS

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Abstract

Bovine Spongiform Encephalopathy (BSE) is a chronic, degenerative disease that affects the central nervous system of cattle, the condition being known as "mad cow disease." BSE is part of the family of transmissible spongiform encephalopathies (TSEs). The main characteristics of TSEs refer to: a) very long incubation period, months or even years; b) progressive neurological disease, often fatal; (c) brain tissue from infected animals showed fibrils associated with scrapie; (d) pathological changes occur only in the central nervous system. Another disease in the EST category is scrapie, which was initially thought to be specific to sheep and does not affect humans, although it was known to be an infectious agent. As there was no other known spongiform encephalopathy at the time of the onset of BSE, it was considered to be derived from scraps, especially given that sheep meat was often served to cows to increase milk production.

Keywords: BSE; cattle; TSE, prions, CJD.

INTRODUCTION

Bovine spongiform encephalopathy (BSE) belongs to the group of transmissible spongiform encephalopathies (TSEs), also known as prion diseases, which are fatal protein-misfolding neurodegenerative diseases. Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases that can occur spontaneously or can be caused by infection or mutations within the prion protein gene PRNP (Kashkevich K. et al., 2007). Transmissible spongiform encephalopathy (TSE) agents or prions induce fatal neurodegenerative diseases in humans and in other mammals.

They are transmissible among their species of origin, but they can also cross some species barriers and induce infection with or without disease in other species. Human TSEs include Creutzfeldt–Jakob disease (CJD), Gerstmann–Stra¨ussler–Scheinker syndrome, Kuru, and fatal familial insomnia. In animals, 4 distinct TSE diseases are recognized: scrapie in sheep and goats, transmissible mink encephalopathy (TME) in mink, chronic wasting disease (CWD) in cervids, and bovine spongiform encephalopathy (BSE) in cattle. BSE is transmissible via BSE-contaminated feed to felines (feline spongiform encephalopathy, FSE) and exotic ungulates (exotic ungulate encephalopathy, EUE) (Richt J.A et al., 2007). These chronic diseases are associated with the accumulation of a protease-resistant (Eraña H. et al., 2020) disease associated isoform of the prion protein (PrP) in the central nervous system and other tissues, depending on the host species (Greenlee Justin J.J et al., 2012). The prion protein (PrP) is encoded by PRNP gene, in brain and other tissues. Although the exact function of this infectious protein is unknown, they are derived from natural cellular proteins (PrPc), which are thought to play a role in natural synaptic function (Collinge J. et al., 1994) or in the outgrowth and survival of neurites (Chen S. et al., 1994). The physiological PrPc will shift the pathological PrPsc (scrapie like prion proteins), which is contagious, to its normal conformation (Kim M.O. et al., 2018). The abnormal types of prion protein (PrPsc) fold into insoluble amyloid, are extremely resistant to protease metabolism, and can cause normal prion (PrPc) conformational shift. Therefore, if a mutation in the prion gene contributes to the development of PrPsc, a prion disorder may be hereditary and it can be contagious if exposure to PrPsc causes the PrPc of the host to undergo conformational shift (MacKnight C., 2001).

Several known forms of PrP have been identified, but is known that prion protein (PrP) plays a central role in the pathogenesis of

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neurodegenerative diseases such as the bovine spongiform encephalopathies (BSE) in cattle, scrapie in sheep, and Creutzfeldt–Jakob disease in humans (Prusiner S.B., 1989). PrP are practically host-coded proteins that have undergone conformational changes and have biological and physicochemical characteristics that differ significantly from those of other infectious agents. For example, they are resistant to inactivation processes that are effective against conventional viruses including those that alter nucleic acid structure or function according to Prusiner's generally accepted hypothesis.

RESULTS AND DISCUSSIONS

Typically, TSEs are acquired through exposure to infectious material, but inherited and spontaneous TSEs also occur. All TSEs share pathologic features and infectious mechanisms but have distinct differences in transmission and epidemiology due the host factors and strain differences encoded within the structure of the misfolded prion protein. So that, the causative agent of TSEs is an infectious prion protein (PrPSc), that is a structurally abnormal and protease resistant isoform of the prion protein (PrP) (Belay E.D., 1999). PrPSc is derived from the endogenous cellular prion protein (PrPC) that has undergone post-translational modification resulting in conformational changes. PrPC is known to play a role in copper metabolism but its normal function(s) in cells are not well defined (Choi S. et al., 2011). Practically, in TSE diseases, the normal cellular protein, PrPc, is converted to abnormal prion protein, PrPSc, which exhibits increased beta sheet content, a change that may drive the additional changes in solubility and protease resistance. Unlike normal cellular protein, PrPSc is relatively insoluble in detergents, is relatively resistant to proteases and is capable of causing a conformational change in additional molecules of PrPc (Richt J.A. et al., 2007). The incidence of TSEs, also referred to as prion diseases, in a defined host population is influenced by a variety of factors. In the individual animal, however, the disease is always associated with an increase of the protease-resistant form of the cellular prion protein, which is then denoted scrapie-associated prion protein (PrPsc) (Prusiner S.B., 1998). In cattle, BSE in cattle is usually thought to be caused by the ingestion of meat and bone meal from scrapie-infected sheep or cattle infected with BSE. The ingestion of meat and bone meal (MBM) produced from scrapie-infected sheep or from cattle with BSE represents the most likely cause of the large BSE outbreak in cattle in the United Kingdom (Eddy R.G., 1995). The function of the physiological prion protein isoform (PrPc) has not yet been elucidated. Affected animals display changes in temperament, abnormal posture, incoordination and difficulty in rising, decreased milk production, and/or loss of body weight despite continued appetite. BSE-affected cattle undergo progressive nervous system degeneration. Animals affected can exhibit changes in temperament, posture and movement abnormalities, and changes in sensation. More precisely, there are signs of anxiety, nervousness or aggression, incoordination, especially hind-limb ataxia, tremor and rising difficulty, and sound and touch hyperesthesia. Additionally, despite continued appetite, many animals have reduced milk production, loss of body condition, or both. There is no cure, and the cattle affected die. The time of incubation varies from 2 to 8 years. The animal 's condition gradually deteriorates after the onset of clinical symptoms, until the animal becomes recumbent, dies, or is killed. Usually, this takes between 2 weeks and 6 months (Detwiler L.A. et al., 2000). The majority of cases in Great Britain occurred between the ages of 3 and 6 years in Holstein Frisian dairy cows (Wilesmith J.W. et al., 1992).

It has been proposed that PrPc plays a role in normal synaptic function (Collinge J. et al., 1994) or in cell-cell interactions and acts as an anti-apoptotic signaling molecule (Premzl M. et al., 2007). BSE was first diagnosed among Holstein/Friesian cattle in the United Kingdom (Wells G.A. et al., 1987) and has since been detected in other countries as well.

From the nearly first cases, the brains of two cows exhibiting neurological symptoms were examined at the United Kingdom Central Veterinary Laboratory in November 1986 by two neuropathologists who noted lesions similar to those usually found in scrapie-affected sheep brains, i.e. spongiform tissue changes (Wells G.A. et al., 1987). Some British researchers have, after collecting epidemiological data, linked the possible cause of the disease to certain animal proteins found in bovine feed. The disorder was diagnosed as a prion disease and called bovine spongiform encephalopathy (BSE) due to its resemblance to scrapie and unusual histopathological symptoms, spongiform tissue changes. As the new variant CJD (vCJD) in humans is likely the result of infection with BSE prions found in meat products from BSE-infected cattle, BSE poses a threat not only to cattle but also to humans (Bruce M.E. et al.,1997; Scott M.R. et al.,1999; Asante E.A et al., 2002).

Also, in some TSE, there is also potential for horizontal transmission, which simply means transmission directly from one animal to an adjacent animal in the herd. However, unlike scrapie in sheep and CWD in deer where horizontal transmission has been shown (Gough K.C. et al., 2010), there is no evidence of horizontal transmission of BSE in cattle. Another proposed route of disease exposure is termed vertical transmission, which is best explained as transmission mainly from parents to offspring either in utero or through birth or lactation. While the possibility of vertical transmission has not been entirely excluded in cattle, it is considered to be very low in incidence if at all, and there is no evidence of prion transmission in milk (Everest S.J. et al., 2006) by ELISA detection, embryos or semen (Wrathall A.E. et al., 2002) via histopathology, immunohistochemistry, or bioassay detection.

Until 2004, TSE disease in cattle was believed to be caused by a single prion strain, classical BSE (BSE-C or cBSE). This conclusion was based on classical strain typing in mice (incubation time, lesion profiles, patterns of PrP staining in the brain) and biochemical features of the proteinase K (PK) resistant PrP (PrPTSE) in natural and experimental BSE, which showed consistent results from all cattle isolates. However, two atypical BSE agents have been reported in 2004 – H-BSE (Biacabe A.G. et al., 2004) and L-BSE (Casalone C. et al., 2004). These variants seem to be sporadic, and all occurred in animals 8 years and older (Langeveld J.P.M. et al., 2011) and can be distinguished from classical BSE by its pathological, molecular and biological phenotype (Konold T. et al., 2014) by the electrophoretic positions of their protease-resistant PrPTSE isoforms (Wilson R. et al., 2012). Indeed, PrP is a glycoprotein and has two sites for the attachment of N-linked glycans, which depending on their utilisation will produce di-, mono-, and unglycosylated PrP. BSE-H PrPTSE shows a significantly higher molecular weight unglycosylated PrP isoform by immunoblot when compared with BSE-C PrPTSE. Similarly, BASE has a slightly lower molecular size than BSE-C PrPTSE and a clearly different glycoform pattern. Furthermore, following transmission into transgenic mice that overexpress the bovine prion protein, both BASE and BSE-H show neuropathological and molecular phenotypes which are distinct from BSE-C (Béringue V. et al., 2007). However, interestingly a recent study has found that survival times are similar in transgenic mice that overexpress the bovine prion protein challenged with either BSE-C or BSE-H (Torres J.M. et al., 2011). BSE-H and BASE were originally described in France (Biacabe A.G. et al., 2004) and Italy (Casalone C. et al., 2004) respectively, however have since been documented in other European countries (Jacobs J.G. et al., 2007), Japan (Hagiwara K. et al., 2017) and North America (Richt J.A. et al., 2007). While BSE-C is thought to be the result of feeding cattle prion-contaminated meat and bone meal, the origin of BASE and BSE-H remains unknown [28].

Resistance to cBSE in cattle has been found to be modulated by 2 nucleotide polymorphisms in regulatory regions of the prion gene (PRNP) (Sander P. et al., 2004; Juling K. et al., 2006; Seabury C.M. et al., 2004). The first is an insertion-deletion (indel) in the promoter region, where the 23-bp deletion removes a binding site for the repressor protein RP58. The second polymorphism is an indel in the first intron, where the 12-bp deletion removes a binding site for transcription factor SP1. Insertion variants of either regulatory element have the potential to lower host prion protein expression levels (Sander P. et al., 2004), thus providing a biological basis for BSE resistance in cattle homozygous for the presence of the insertions. H-type BSE has been described in cattle from France (Biacabe A.G. et al., 2004), Germany (Buschmann A. et al., 2006), Japan (Sugiura K. et al., 2009), the Netherlands (Biacabe A.G. et al., 2007), Poland (Jacobs J.G. et al., 2007), Switzerland (Tester S. et al., 2009), the United Kingdom (Stack M. et al., 2009), Canada (Dudas S. et al., 2010), United States (Richt J.A. et al., 2007) and Sweden (Gavier-Widen D. et al., 2008). The molecular phenotype of the H-type BSE cases is characterized by a higher molecular mass of the unglycosylated PrPSc isoform and a strong labeling of all 3 PrPSc polypeptides.

There are some genetic variations of PRNP gene in cattle, so that several experiments have been carried out in cattle to find such a relationship between BSE and cattle genome polymorphisms (Goldmann W. et al., 1990; Neibergs H.L. et al., 1994; Heaton M.P. et al., 2004).

Genetic variations in the prion protein gene (PRNP) are linked to the occurrence of transmissible spongiform encephalopathies (TSEs) also called prion diseases in humans, sheep and mice. It has been reported that single nucleotide polymorphisms (SNPs) of PRNP have various effects on the susceptibility and incubation time of prion diseases in humans, mice, and sheep (Baylis M. et al., 2004). In sheep, polymorphisms in codons 136, 154, and 171 are correlated with susceptibility to scrapie and can

thus be used to control disease incidence (Hills D. et al., 2001) whereas in humans, a polymorphism in codon 129 has a critical influence to variant CJD incidence (Wadsworth J.D.F. et al., 2004).

The prion gene, *PRNP*, in cattle is located on the forward strand of chromosome 13, from 47,400,413 to 47,418,507 base pairs (bp), within syntenic group U11 (Ryan A.M. et al., 1993) and mapped on BTA13 chromosome (13q17) (Schläpfer J. et al., 1999). This gene extends over 20.2 kb, and full-length mRNA containing three exons is 4244 bp. Exon 1 spans 53 bp and exon 2 spans 98 bp (Inoue S., et al., 1997). The size of the second intron has been estimated to be approximately 14 kb (Horiuchi M. et al., 1998). The whole ORFis located within exon 3 and has a size of 795 bp (Yoshimoto J. et al., 1992). The complete genomic sequence of 78 056 bp has also been determined and deposited in the EMBL/GenBank database under accession number AJ298878 (Hills D. et al., 2001). In cattle, the prion gene consists of three exons, as compared to the human gene which is composed of two exons. However, consistent in all orthologs across species, only the last exon is translated to a protein. There are three genes within this chromosomal locus, the prion gene *PRNP*, the doppel gene *PRND*, and the testis-specific alternatively spliced transcription product *PRNT* (Murdoch B.M. et al., 2015). The doppel gene *PRND*, also called prion-like gene (prion protein 2), is located immediately downstream (47,444,352–47,449,390 bp) of the *PRNP* gene. Interestingly, the doppel protein, despite its genomic proximity to the *PRNP* gene is not expressed at appreciable levels in the brain; however, it is expressed in the testis and in fact its absence in the testis results in sterility. A third member of this gene locus is prion protein testis–specific *PRNT* found immediately downstream to *PRND* but on the reverse strand. Expression of the *PRNT* gene is exclusively found in the adult testis in human, rhesus monkey, and sheep; however, it is not observed in mouse, rat, and cow (Premzl M. et al., 2007).

In addition to the three previously described genes, another gene with a role in prion disease has been discovered. Shadow of prion protein homolog and its gene *SPRN* has been mapped to the reverse strand of chromosome 26, from 25,812,626 to 25,813,057 bp in cattle. Importantly, in addition to the prion gene, *SPRN* has been implicated in prion-related disease susceptibility. Specifically, *SPRN* has been associated with prion disease in cattle (Uboldi C. et al., 2006; Gurgul A. et al., 2012) and humans (Beck J.A. et al., 2008).

SPRN is expressed in high levels in the brain and at lower levels in testis. Due to the fact that *SPRN* is more conserved than *PRNP*, it has been hypothesized that it is the ancestor gene of a duplication event. The model proposes that the duplication of *SPRN* gave rise to *SPRNB*, which then resulted in the *PRNP* gene cluster (Beck J.A. et al., 2008). While there is homology across these genes and some suspected redundancies, the full extent and significance of these relationships have yet to be fully characterized and, of course, merits further study. Again and somewhat unique to prion diseases, an animal cannot accumulate misfolded proteins, the hallmark of TSE disease, if they do not have that protein to begin with. Therefore, the native prion protein is actually required and essential for the development and progression of TSE disease (Weissmann C. et al., 2003) and genetic variations in the prion gene have been associated with TSE susceptibility in humans (Prusiner S.B., 1998), (Gambetti P. et al., 2003), sheep (Laegreid W.W. et al., 2008) and deer (Blanchong J.A. et al., 2009), (Wilson G.A. et al., 2009). Although amino acid differences in the prion protein are a major contributor to susceptibility and/or resistance risk factor in humans (Spudich S. et al., 1995) and sheep (Baylis M. et al., 2004), this is not the case for cattle. Bovine codon E211K, analogous to codon E200K in human CJD, has only been observed twice in cattle, the first of these cases is associated to atypical BSE and the other case is the offspring of the first (Heaton M.P. et al., 2004; Nicholson E.M. et al., 2008).

The bovine prion gene contains more than 390 SNPs in the 25-kb region of chromosome 13 containing the *PRNP* gene. This chromosomal segment contains distinct regions of high and low LD (Linkage Disequilibrium) that is conserved across many *B. taurus* cattle populations (Clawson M.L. et al., 2006).

The region of high LD includes the promoter region, exons 1 and 2, and part of intron 2 (6.7 kb) of the *PRNP* gene. A 23-bp insertion/deletion (indel) polymorphism in the promoter contains a binding site for the repressor protein 58 (RP58) and a 12-bp indel in intron 1 has a binding site or the transcription factor specificity protein 1 (SP1). The presence or absence of these binding sites modulate the expression of PRNP and possibly the expression of PrP in species. Expression of the cellular PrP is necessary for the transmission and propagation of prion diseases (Montrasio F. et al., 2000). Based on reporter gene assays, increased level or PrP decreases the incubation period for cBSE (Sander P. et al., 2005). Susceptibility or resistance to TSE, is associated with variations in

the non-coding region of PRNP, including the promoter and/or enhancer. Nucleotide changes in the non-coding region may affect mammalian PrPC expression, which is also known to affect TSE susceptibility or resistance (Sander P. et al., 2004). These polymorphisms are a 23-bp indel at position −1594 and a 12-bp indel at position +300 (the positions of the polymorphisms are given with respect to the transcription start site in GenBank accession No. AJ298878); both are associated with promoter activity and bovine PrPC expression levels in vitro (Sander P. et al., 2005). Therefore, these polymorphisms can both seemingly influence BSE incubation times and susceptibility. So, both the 23- and 12-bp indels that have been associated with C-BSE susceptibility are contained in this region of high LD (Sander P. et al., 2004; Vernerova K. et al., 2014). Several studies have shown the impact on BSE sensitivity in cattle of a 23-bp insertion-deletion (indel) polymorphism located 1.6 kbp upstream of exon 1, and a 12-bp indel inside intron 1 (Haase B. et al., 2007). Although it is clear that cattle have both been substantially correlated with BSE with the -/-23 bp promoter genotype and the -/- 12 bp Intron 1 genotype, there is no agreement about which genotype is most closely linked to BSE (Sander P. et al., 2004). In addition, indel polymorphisms affecting the sensitivity of classical BSE tend not to be relevant in cattle to other transmissible spongiform encephalopathies (Brunelle B.W. et al., 2007). To date, in some cattle in Asia (Nakamitsu S. et al., 2006), Europe (Juling K. et al., 2006) and United States of America (Seabury C.M. et al., 2004), the frequency of polymorphism in the PRNP gene promoter region has been identified. Due to their poor milk and meat output, the number of local cattle breeds in Turkey has been declining.

On the other hand, on various platforms, their high tolerance to diseases and parasites is rated (Bakır G. et al., 2003). For example, according to Imran et al [74], Pakistani cattle are relatively more resistant to classical BSE than European cattle. However, the key risk factor for classical BSE is the dietary exposure of susceptible cattle to contaminated feedstuffs.

The reminder of *PRNP*, including the entire coding region, has relatively low LD. To account for the genetic architecture of the *PRNP* gene, a set of haplotype-tagging single-nucleotide polymorphisms (htSNPs) has been described that efficiently define haplotypes within and across each of the LD regions (Clawson M.L. et al., 2006). These SNPs were used to test for association between *PRNP* haplotypes and susceptibility to either C-BSE or atypical BSE susceptibility (Murdoch B.M. et al., 2010). Susceptibility or resistance to a TSE disease can be influenced by at least 3 factors related to the host prion protein: protein expression levels, the number of octapeptide repeats, and specific amino acid differences. These 3 factors are all relevant to prion biology in cattle. Non-coding region polymorphisms in cattle have been identified that modulate expression level and influence susceptibility to cBSE (Sander P. et al., 2004; Juling K. et al., 2006) but not atypical BSE (Brunelle B.W et al., 2007).

The presence of additional octapeptide repeats in transgenic mice (Castilla J. et al., 2005) and Brown Swiss cattle (Sauter-Louis C. et al., 2006) (have been reported to result in increased susceptibility to classical BSE. Amino acid differences are a major component in susceptibility and resistance to acquired TSE disease in sheep and are the basis for genetic TSEs in humans (Mead S., 2006).

However, studies in cattle revealed that regions outside of the open reading frame are associated with variation in disease susceptibility. While a few studies identified the octapeptide-repeat region, (Sander P. et al., 2005) genetic analyses primarily identified two insertion/deletions in promoter regions of the prion gene, (Vernerova K. et al., 2014) associated with BSE susceptibility and/or resistance. For example a 23-bp insertion/deletion (indel) polymorphism in the promoter contains a binding site for the repressor protein 58 (RP58) and a 12-bp indel in intron 1 has a binding site or the transcription factor specificity protein 1 (SP1). A study conducted by (Murdoch B.M. et al., 2015) examined these SNPs and the 12-bp and 23-bp indels to test *PRNP* haplotypes for an association with C-BSE in 330 European Holstein cows from the U.K. BSE epidemic, of which 146 were BSE cases and 184 were controls. A combination of sequencing, SNP assay (Illumina goldengate assay), and polymerase chain reaction amplification was used to genotype 18 SNPs and 2 indels in 95 BSE case and 134 control animals (Murdoch B.M. et al., 2010).

The presence or absence of these binding sites modulate the expression of PRNP and possibly the expression of PrP in species. Expression of the cellular PrP is necessary for the transmission and propagation of prion diseases (Montrasio F. et al., 2000). Based on reporter gene assays, increased level or PrP decreases the incubation period for cBSE (Sander P. et al., 2005). Furthermore, that two bovine *PRNP* alleles have been associated with susceptibility to C-BSE (classic BSE): a 23-

bp deletion within the promoter region and a 12-bp deletion within intron 1 (Sander P. et al., 2004; Juling K. et al., 2006; Hills D. et al., 2001; Vernerova K. et al., 2014).

However, the deletion alleles are not entirely independent of one another as there is high linkage disequilibrium (LD) between the two polymorphic sites in *Bos taurus* cattle populations. This suggests that the possible effects of variations in the *PRNP* gene on incidence of C-BSE may be better understood if *PRNP* haplotypes were considered in testing for association with disease incidence. Moreover, *PRNP* haplotypes, containing one or both of the two insertion/deletion alleles, may have a stronger association with either susceptibility or resistance to C-BSE than if the insertions and deletions (indels) are considered independently.

CONCLUSIONS

Modifications in the prion protein cause prion diseases such as scrapie in sheep, BSE in cattle, and CJD in humans. As is the case with prion diseases, pathogens that infect multiple species can leap species boundaries and impact endangered species. It is known that there is no preventive therapy in other species for BSE in cattle or for prion diseases. Therefore, in the cattle population, genetic selection is a special tool for eradicating BSE. The only sustainable solution is using in reproduction only those individuals that are resistant to BSE. This could be a good strategy for eradicating BSE.

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A REVIEW: DNA MARKERS ASSOCIATED WITH PRODUCTION TRAITS IN DIFFERENT CATTLE BREEDS

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Abstract

The main objective of modern livestock breeding is to find an efficient and a fast way to increase productivity and quality, and the application of technologies based on DNA markers have a great influence on the livestock and also contributes further to the mapping of the genomes of different species of economic importance. Past decade research carried out at the DNA level had a main goal: to identify the genes responsible for the expression of quantitative characters and the detection of places of interest, the latter becoming markers of DNA or SNP type that can be used in the selection process and improvement programs of dairy farms.
In Romania, several studies were performed in order to identify the associations of the genetic markers with the main traits of milk production by Vlaic *et al*, (2001; 2003; 2005); Creangă *et al*., (1996, 2002, 2003; 2007, 2008, 2010); Bâlteanu *et al*., (2007a; 2007b, 2008; 2010a; 2010b, 2013); Bugeac et al., (2013a, 2013b, 2013c, 2015, 2019), respectively for the quality of meat by Carşai *et al*, (2009, 2010, 2013).

Key words: (DNA markers, dairy milk, beef, cattle, polymorphism)

INTRODUCTION

Several studies to date have shown significant associations between a series of mutations in the coding or non-coding regions of DNA (genetic markers) and diseases with genetic substrate or phenotypic characters of interest (external or production). The explanation for this phenomenon is related to the fact that mutations can affect the expression or the expression product of the genes that control these characters. In cattle, more than 80% of the qualitative traits are determined by autosomal recessive genes (Healy *et al,* 1996). Genomic association studies aimed at decoding the genetic substrate of quantitative traits and highlighted the involvement in their genetic determinism of several chromosomal regions containing functional genes. Using the information provided by SNP markers, markers for milk production were identified in cattle (Mai *et al*, 2010; Cole *et al*, 2011), markers for meat production (Casas *et al*, 2000; Casas *et al*, 2001; Snelling *et al*, 2010; Snelling *et al*, 2011.

Genetic markers associated with beef production in cattle.

The candidate genes for quantitative character loci for milk and meat production are selected based on the associations that are established between biochemical or physiological processes and the respective quantitative character. Subsequently, these loci are tested as loci of a quantitative character (QTL). The aim is to identify DNA markers positively associated with different palatability characteristics. For example, genes specifying thyroglobulin, leptin, and calpastatin have been shown to influence the tenderness, juiciness, and marbling of meat, carcass quality, and body weight (Carşai *et al*, 2010).

Calpastatin (CAST) - is located on bovine chromosome 7 and is responsible for the meat tenderness, this being one of the essential criteria for the selection of beef cattle, because this trait is desired by consumers. Studies have shown that calpastatin is one of the main factors that higly associated with fragility of meat because it regulates the activity of m-Calpain and µ-Calpain enzymes. These proteolytic enzymes are responsible for the breakdown of muscle fiber, producing post-mortem tenderness of the meat (Koohmaraie *et al*, 1996). Significant associations with meat tenderness have been identified in several breeds of cattle (Barendse *et al*, 2007).

µ-Calpain (CAPN1) is a proteolytic enzyme of skeletal muscle, it is encoded by the CAPN1 gene located on chromosome 29. In *Bos taurus*, an SNP associated with meat tenderness has been

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identified (Page *et al*, 2002). Other SNPs associated with this trait have been identified in *Bos indicus* (Riley *et al*, 2003; White *et al*, 2005).

Myostatin (MSTN). The myostatin gene, located on bovine chromosome 2, has been identified as being responsible for the genetic determinism of this trait and is responsible for muscle hypertrophy in cattle. Phenotypically speaking, animals with two copies of the mutant allele responsible for the double croup have a higher birth weight (McPherron *et al*, 1997). Studies have shown that animals that are homozygous have difficulty giving birth as opposed to heterozygous ones (Casas *et al*, 1999). Double-legged animals have been shown to have 30% more muscle mass than others. The flesh of these animals is also much tenderer (Arthur, 1995). This marker is currently used in selection programs for the production of heterozygous individuals.

Thyroglobulin (TG) - is a glycoprotein hormone synthesized by the follicular cells of the thyroid where it is stored and which is subsequently iodinated, whose coding gene is located on bovine chromosome 14. Thyroglobulin is the carrier of triodothyronine (T3) and thyroxine (T4) stored in the lumen of the gland, so that T3 and T4 have been associated with the degree of meat marbling in Wagyu cows. TG5 polymorphism is located in the leader sequence at the 5 'end of the thyroglobulin gene and has been associated with intramuscular fat content in cows fed ad libidum (Carşai *et al*, 2010). Cows homozygous or heterozygous for the T delta allele (CT or TT) have a much higher degree of marbling than those homozygous for the C delta allele (Barendse, 1997). Individuals with the T allele in the genotype have superior growth performance and a higher degree of meat marbling. The selection assisted by this marker will not aim at a lower amount of subcutaneous fat but at a higher degree of marbling of the meat.

Leptin (Lep) - is a hormone synthesized in adipose tissue and secreted into the blood, with implications for regulating body weight, bone formation, fertility and immune functions of the body. Leptin, called the obesity gene is associated with risk factors in cardiovascular disease, infertility, etc. and is located on bovine chromosome 4. This hormone also plays an important role in glycogen synthesis, glucose transport and muscle lipid disposition (Carşai *et al*. 2010), and the larger the adipocytes, the more leptin mRNA will be present.

Genetic markers associated with milk production in cattle

Polymorphisms within key gene structure, such as: pituitary growth factor (PIT1), somatotropic growth hormone (GH), insulin-like growth factor 1 (IGF1), prolactin (PRL), prolactin receptor (PRLR), growth hormone receptor (GHR), signal transmission and transcription activation factor 5 (STAT5), AcylCoA diacylglycerol acyltransferase 1 (DGAT1), alpha-lactoalbumin (LALBA) may have a positive or negative effect on the development of the mammary gland, its capacity lactogenic and implicitly on the quantitative and qualitative production of milk. The effects of some polymorphisms identified in some of these loci on the quantity and quality of milk have been evaluated in several studies performed on various breeds of cattle. For example, the SNPs of the diacylglycerol acyltransferase 1 (DGAT1), stearoyl-CoA dasaturase 1 (SCD1), fatty acid synthetase (FASN), lipoprotein receptor (OLR1), prolactin (PRL), signaling factor 5 and activation genes of transcription 5A (STAT5A) growth hormone receptor (GHR) are closely correlated with the composition of cow's milk in several breeds (Mele *et al*, 2007; Moioli *et al*, 2007; Schennink *et al*, 2009; Sun *et al*, 2009).

Pituitary growth factor (PIT1 or POU1F1) is a transcription factor synthesized in the hypothalamus (Moody *et al*, 1995). The gene encoding the 291 amino acid protein is located in cattle on chromosome 1. PIT 1 plays an important role in activating in the anterior pituitary gland the expression of genes encoding prolactin and growth hormone (Bona *et al*, 2004). PIT1 is also involved in regulating the expression of the gene encoding the beta subunit of thyrotropin-releasing hormone (TSH) - a hormone essential for the activity of the thyroid gland. Polymorphisms in the structure of the PIT1 gene can lead to deficiencies in the synthesis of growth hormone, prolactin, and thyrotropin-releasing hormone (Radovick *et al*, 1992). Mutations in the structure of the PIT1 gene can have positive or negative influences on milk production, which is why it is currently considered an extremely valuable genetic marker in improving animal production.

Similar studies indicate the same associations between PIT1 A and milk production, respectively the percentage of milk protein, but also with a lower percentage of fat (Zwierzchowski *et al*, 2002; Mattos et al., 2004 Hori-Oshima et al., 2003). However, there are a number of studies (Dybus *et al*, 2004; Zakizadeh *et al*, 2007; Trakovická et al., 2014) performed on different breeds of cattle in which no positive associations between PIT1-Hinf1 polymorphisms and the productive properties of milk.

Somatotropic hormone (GH) is synthesized and secreted by somatotropic cells in the anterior

pituitary gland under the influence of PIT1 (Bona *et al*, 2004). In cattle, the gene encoding GH is located on chromosome 19 and encodes a 191 amino acid protein (Hediger *et al*, 1990). It has a role in stimulating cell division and differentiation as well as an essential role in regulating metabolism. GH stimulates the secretion of secretory epithelial cells from the mammary gland by activating in the liver the gene encoding insulin-like epithelial growth factor 1 (IGF1). Experiments performed on cattle have shown that exogenous administration of GH increases the amount of milk by 10-15% (Zhou *et al*, 2008) by stimulating the proliferation of lactogenic breast epithelium. This is done indirectly by stimulating the expression of the IGF1 gene in the liver. GH also acts directly in the mammary gland by binding to its receptor on the secretory mammary epithelial cell (GHR). It has a stimulating effect on genes that are involved in the synthesis of major proteins in milk, by their transcriptional activation mediated by STAT5 factor. On the other hand, by stimulating the synthesis of α-lactoalbumin by the LALBA gene, there is an increase in the amount of the enzyme lactose - synthetase, the enzyme involved in the synthesis of lactose. Therefore, there is an increase in the amount of milk because the proportion of lactose depends on the amount of water absorbed from the cytoplasm during the synthesis of milk droplets (Yang *et al*, 2005; Zhou *et al*, 2008). Some polymorphisms significantly associated with milk production have been reported at the GH gene locus. Exon 5 identified a G / C type polymorphism located at the restriction site of the AluI enzyme and which causes the substitution of a leucine (L) with a valine (V) at position 127 of the mature protein (Lucy *et al*, 1993; Zhang *et al*, 1993). Dybus (2002) studied in the Polish Frisian breed the associations between this polymorphism from exon 5 and milk production. The LL genotype was associated with a higher amount of fat and protein. Yardibi *et al*, (2009) studied the same polymorphism in South Anatolian and East Anatolian Red bull breeds. In both breeds the LL genotype was associated with a higher amount of milk and a higher percentage of fat compared to the other two genotypes. In contrast, in other studies, the VV genotype was associated with a higher amount of milk and a better composition (Zwierzchowski *et al*, 2002).

Insulin-like growth factor 1 (IGF1) is a protein hormone consisting of 70 amino acids with a molecular weight of 7.5 kDa, being similar to insulin; the gene encoding it is located in cattle on pair 5 chromosomes. IGF1 is produced mainly in the liver by the direct action of GH, being the primary mediator of the effects of this hormone in tissues. IGF1 is one of the most potent activators of stimulating cell growth and proliferation and a potent inhibitor of apoptosis (cell death). It is produced throughout its life, reaching its maximum level during puberty. Its effects of stimulating cell division and differentiation have repercussions on every cell in the body. IGF1 is also produced locally in the mammary gland (through the direct action of growth hormone), where it stimulates together with estrogen hormones the development of galactophore channels and the growth of the mammary epithelium by stimulating cell divisions and inhibiting apoptosis (Plath-Gabler *et al*, 2001). At the IGF1 gene locus, Siadkowska *et al*, (2006) studied in the Polish Holstein breed the associations between a C472T-type polymorphism located in the 5 'untranslated region of the gene and milk production. The CT genotype was significantly associated with a higher amount and percentage of fat and protein in milk compared to the CC genotype. Mehmannavaz *et al*, (2010) studied the effect of the same polymorphism in the Holstein Iranian breed. The CT genotype was associated with a higher amount of milk and fat compared to the other genotypes.

Prolactin (PRL), also known as luteotropic or lactotropic hormone (LTH), is a protein hormone made up of 199 amino acids with a molecular weight of 24kDa; the gene encoding it is located in cattle on the chromosomes in the pair 23. PRL is synthesized and secreted by lactotropic cells in the anterior pituitary lobe, but is produced in small amounts and in other tissues including the mammary gland (Le Provost *et al*, 1994). Together with GH, PRL stimulates the growth and development of breast tissue, triggering and maintaining lactation and the synthesis of milk components by regulating the expression of genes encoding the 6 major proteins in milk and genes involved in the synthesis of lactose and fats. The increase in serum concentration of PRL circulating during pregnancy, leads to an increase and differentiation of the lactogenic tissue in the mammary gland, which is correlated after birth with an increase in milk production. Activation of the gene encoding PRL is done under the influence of the transcription factor PIT1, which binds to the gene promoter and thus promotes its expression in the pituitary gland. Several polymorphisms have been identified at the PRL gene locus, of which an A / G type substitution in exon 4 leads to a restriction site for the RsaI enzyme (Lewin *et al*, 1992; Mitra *et al*, 1995). Dybus *et al*, (2004) studied in the Polish Frisian breed the associations between this polymorphism and milk production, the AA genotype being associated with a higher percentage of protein. Brym *et al*, (2005a) studied

the associations between an A8398G polymorphism in exon 4 and milk production in the Polish Holstein and Jersey breeds. The AG genotype was associated with a higher amount of milk while the GG genotype was associated with a higher percentage of protein in milk. Miceikiene et al. (2006) studied the polymorphism of the prolactin gene in four cattle breeds in Lithuania. Genotype AA was associated with a higher percentage of fat in milk compared to genotypes AB and BB. Ghasemi et al, (2009) studied the same RsaI type polymorphism from exon 4 in the Montbeliard breed, the AA genotype being associated with a higher amount of milk. Similar results regarding the milk fat percent were reported also for Chinese Holstein (Echeverry *et al*, 2011; Lewin *et al*, 1992; Chung *et al*, 1996; Dybus, 2002; Brym *et al*, 2005) and Russian Red Pied cattle (Alipanah *et al*, 2007).

The prolactin receptor (PRLR) and the *growth hormone receptor (GHR)* are trans-membrane proteins with an essential role in the uptake and further transmission of prolactin and growth hormone signals to STAT5 family cytoplasmic transcription factors. The genes encoding the two receptors are located in cattle on chromosomes in pair 20 in the proximity of each other (Georges *et al*, 1995; Arranz *et al*, 1998). These two genes are specifically expressed in all target tissues where the two hormones act, especially in the mammary gland in the case of the prolactin receptor, respectively in the liver and in the mammary gland in the case of the growth hormone receptor. Both receptors have three domains: extracellular (through which it binds to hormones whose effect mediates it), transmembrane and intracellular (through which it interacts with STAT5A factor). Binding of prolactin and growth hormone to their specific receptors causes their homodimerization, leading to phosphorylation activation of STAT5A factor that has intracellular localization (Herrington *et al*, 2001). At the locus of the PRLR gene, Brym *et al*. (2005b) studied the associations between the Polish Holstein and Jersey breeds between an A205C polymorphism from intron 9 and milk production. The CC genotype was associated with a higher amount of milk and a higher percentage of protein compared to AA and AC genotypes. Viitala *et al*, (2006) studied a polymorphism that causes the substitution of a serine (S) with an asparagine (N) at position 18 of the prolactin receptor in the Finnish Ayrshire cattle, this polymorphism being associated with a higher amount of fat and protein in milk.

At the GHR gene locus, the existence of polymorphisms with a marked effect on milk production has been suggested in various studies (Georges *et al*, 1995; Arranz *et al*, 1998). In particular, an exon 8 T / A polymorphism, which results in the substitution of a phenylalanine (Phe) with a tyrosine (Tyr) at position 279 of the mature protein, has been significantly associated with this character. In the Friesian and Jersey Holstein breeds this polymorphism was significantly associated with a higher percentage of protein and fat and to a lesser extent with a higher amount of milk (Blott *et al*, 2003). Viitala *et al*, (2006), respectively Sun *et al*. (2009), studied this polymorphism in the Finnish Ayrshire and Holstein breeds in China, respectively. In the Finnish Ayrshire breed, the presence of Tyr (allele A) was associated with a higher percentage of protein and fat (Viitala *et al*, 2006), and in Holstein with a higher percentage of protein (Sun *et al*, 2009).

Signal transmission and transcription activation factor 5 (STAT5) is an intracellular protein first identified in the lactating mammary gland, and later identified in other tissues. Two protein forms of this factor have been identified called STAT5A and STAT5B, which have a homology of 93% in terms of amino acid sequence. They are encoded by two extremely similar genes in structure (Darnell *et al*, 1997) and located in close proximity to each other on the chromosomes in pair 19 of taurine (Seyfert et al., 2000). The gene encoding STAT5A factor is expressed at all stages of mammary gland development and shows few changes in its expression profile at these stages. In the epithelial cell of the mammary gland, the factor STAT5A has a double role. It acts as an essential intracellular mediator of the transmission of prolactin signals in the cell nucleus, where by binding specific regions of the promoters of genes encoding major proteins in milk determines their transcriptional activation (Wakao *et al*, 1994). It is also the main mediator of the action of growth hormone on some target genes (Argetsinger *et al*, 1996). It mediates under hormonal influence and transcriptional activation of genes involved in the synthesis of fats, lactose and other components of milk. At the locus of the STAT5A gene, Brym *et al*, (2004) studied a G9501A polymorphism in intron 9 in the Jersey breed. The GG genotype was associated with a higher amount of milk and a higher percentage of fat, while the AA and AG genotypes were associated with a higher percentage. higher protein. Flisikowski *et al*, (2004) studied in the Polish Frisian breed a T12743C type polymorphism from exon 16. The TC genotype was associated with a higher amount of milk and a higher percentage of dry matter,

protein and lactose in milk compared to the TT genotype. Sadeghi *et al*, (2009) studied the same polymorphism in the Italian Holstein breed, the CT genotype being associated with a higher amount of protein in milk. Selvaggi *et al*, (2009) studied the Italian Brown breed a C6853T polymorphism from exon 7, the CC genotype being associated with a higher amount of milk and a higher percentage of protein compared to CT and TT genotypes.

AcylCoA diacylglycerol acyltransferase 1 (DGAT1) is a protein with an enzymatic role consisting of a polypeptide chain of 425 amino acids, with a molecular weight of 47 kDa; the gene encoding it is located in cattle on chromosomes in pair 14 (Coppieters *et al*., 1998; Riquet *et al*, 1999). In milk, fats are present in the form of small spherical or elliptical globules made up of triglycerides, which represent 98-99% of total fat. Their synthesis in the lactating mammary gland is catalyzed by the key enzyme DGAT1 (Winter *et al*, 2002). This essential metabolic process takes place in the smooth endoplasmic reticulum where this enzyme uses as substrate for their synthesis diacylglycerol and acetyl coenzyme A. DGAT1 is the main enzyme involved in the synthesis of adipocyte triglycerides (Kuhn *et al*, 2004).

At the locus of the DGAT1 gene, an AA / GC polymorphism was identified in exon 8 which has the effect of substituting a lysine (K) with an alanine (A) in the mature protein (Winter et al., 2002; Kuhn et al., 2003).). The effect of this polymorphism on milk production has been studied in cattle populations in New Zealand (Spelman *et al*, 2002), Israel (Weller *et al*, 2003), the Netherlands (Grisart *et al*, 2004), Germany (Sanders et al. et al., 2006), Poland (Pareek et al., 2005), Simmental in Czech Republic (*Hasunova et al*, 2014), and France (Gautier et al., 2007; Näslund et al., 2008). It has been found that the effects of this amino acid substitution are to increase the amount and percentage of fat in milk. Following an expression study, it was highlighted that the lysine-containing variant has a higher enzymatic activity than the alanine-containing variant (Grisart *et al*, 2004).

ATP-G2 binding box (ABCG2). The ATP G2 binding cassette (ABCG2) is a gene that carries drugs, having mainly a protective function against xenotoxin. Xenotoxins are transferred from mother to infant through milk. The ABCG2 gene is located in a specific quantitative trait (QTL) linkage region for milk production and milk composition, making it a functional candidate gene for associations with milk production traits. The ABCG2 gene is located in the *Bos* genus on chromosome 6.

CSN2 gene encoding Kappa-casein. K-CN is a very important milk protein consisting of 169 amino acids. There are 2 cysteine residues, which can form intra- and inter-molecular disulfide bridges (S-S), giving rise to several polymeric forms. These cysteine residues, under the influence of heat, can also form disulfide bridges with free SH groups of β-lactoglobulin. It is located on chromosome 6 (6q31). K-CN is completely soluble in the presence of calcium ions. It is the only casein that can be associated with a carbohydrate co-factor, the most common carbohydrates with which it is associated are galactose, galactosamine and N-acetylneuraminic acid (Creamer *et al*, 1998). The most common are variants A and B of K-CN. Almost all studies aimed at testing the associations between the genetic variants of K-CN, have shown that the BB genotype is associated with a higher content of milk in total protein and casein compared to the AA genotype: BB> AB> AA (Jakob *et al*, 1994).

Milk from the AE and EE genotypes also showed a less firm curd, which was also found in other breeds: Holstein Friesian breed - BB> AB> BE> AA / EE / AE; Angler breed - BB> BE> AB> EE> AA / AE (Oloffs *et al*., 1992); Fleckvieh breed: BE> AB> AA> AE (Jakob *et al*, 1994); Schwarzfleckvieh breed - AB> AA> AE (Jakob et al., 1994). Lodes *et al*, (1997) found the following order of genotypes regarding clot firmness: BB / BC> AC / AB> AA> BE> AE. The same decrease in clot firmness is found in the case of BG and AG genotypes: BB> AB> AA> BG> AG (Erhardt *et al*, 1993).

As can be seen above, the reports regarding the firmness of the curd are contradictory. Interestingly, the AC and BC genotypes, which have the longest clot formation time, have a coagulation firmness almost as good as the BB genotypes (Lodes *et al*, 1997).

Due to the economic importance of cheeses, the potential for turning milk into cheese is an important improvement objective. Many studies in this direction have been carried out so far. However, it is quite difficult to compare different experiments, which aimed to study the amount of cheese obtained, due to the different protein and fat content of raw milk, experimental conditions and different statistical interpretation of experimental data.

In other more recent studies, the same differences were found in terms of the amount of cheese obtained in favor of the BB genotype. In two Gouda cheese experiments, only a small difference was observed for the conversion of total milk nitrogen into cheese component nitrogen, which was 3% for BB genotypes and 2.7% for AA genotypes (Van den Berg *et al*, 1992).

In an experiment conducted in order to obtain Cheddar-type cheese, 6.2% more cheese was obtained from milk from BB genotypes compared to AA genotype (Fitzgerald *et al*, 1997). In another experiment to obtain Cheddar and Mozzarella cheese, Walsh *et al*, (2008) obtained 5.5% differences in favor of the BB genotype.

CSN2 encoding beta casein. β-CN is a protein consisting of 209 amino acids and is encoded by the CSN2 gene which is located on bovine chromosome 6. This protein has a hydrophobic character, and at room temperature it is sensitive to calcium ions. By the action of plasmin β-CN is cut into 3 positions, giving rise to the 3 γ-caseins. The β-CN family constitutes approximately 45% of the casein in cow's milk and represents a complex polymorphism due to the action of plasmin (Eigel *et al*, 1984) and high genetic variability (Formaggioni *et al*, 1999). At the β-CN locus, the A2A2 genotype was significantly associated with the Canadian Holstein breed with a higher amount of milk but less fat compared to the A1A1 genotype (Ng-Kwai-Hang 2006; Cieslinska *et al*, 2019). In another Holstein study, cows with the A1A1 genotype produced significantly more milk compared to other genotypes (Bovenhuis, 1992). Milk from BB genotypes at the β-CN locus showed the shortest clot formation time, compared to other genotypes: BB <A1B <A2C <A2B <A1A1 <A1A2 / A2A2 (Lodes *et al*, 1997). The other genotypes at this locus differ only slightly in coagulation time, except for the CC genotype which has a significantly shorter time compared to genotypes A1A1, A1A2, A2A2 (Delacroix - Buchet *et al*, 1994). Genotypes at the β-CN locus (with the exception of the CC genotype) do not appear to differ significantly in terms of clot firmness. At standardized pH, milk from CC genotypes was found to form a weak clot, compared to genotypes A1A1, A1A2, A2A2: CC <A1A1 / A1A2 / A2A2 (Delacroix-Buchet et al., 1994). The same seems to be true for the BB genotype. In other experiment, in order to obtain Beaufort cheese from the French Tarentaise breed, the amount of cheese from genotypes A1A1, A1A2, respectively A2A2 was 15% higher than in the case of CC genotype. The C allele, which has a high frequency in this breed (17%), has been associated with a spicy taste of cheese and a harder consistency of it (Delacroix-Buchet et al., 1994).

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PATHOLOGIES OF THE LIVER AND GALLBLADDER AT DOGS AND THEIR IMAGISTICS – STATISTIC ANALYSES

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Abstract

Pathologies of the liver and gallbladder present very diversified clinical signs, needing additional exams, like imagistic ones.
The radiological and ultrasonography exam represent the main imagistic methods for diagnosing this pathologies at dogs. Radiography brings data regarding the shape and volume modification of the liver, without offering any information about the gallbladder. Ultrasonograpy of the liver allows a more detailed examination of the internal structure, including vascularization and the bile ducts. Ultrasonography is also useful in fine needle aspiration and biopsy, as a non-invasive method of diagnosis.
The goal of this study was of analysing the prevalence of liver and gallbladder pathologies arriving at the Faculty of Veterinary Medicine Iași, and their diverisity. A number of 177 pacients were examined between 2018 and 2020. All the dogs undergone the ultrasonographic exam. During the examination, 62 pacients were diagnosed with modifications at the level of the gallbladder while only 43 were diagnosed with modifications at the level of the hepatic parenchima.

Key words: Ultrasonography, liver and gallblader pathologies, prevalence

INTRODUCTION

The liver is the organ situated most cranially in the abdominal cavity, located in direct contact with the diaphragmatic wall. Its surface is in contact with the stomach, duodenum and pancreas. Anatomically, it is localized between the diaphragmatic wall, the stomach, the right kidney and the cranial portion of the duodenum.

The gallbladder is a pear-shaped like organ, with liquid content, situated at dogs between the medial hepatic lobe and the quadrate lobe. It stores the bile and it secrets it through the bile ducts into the duodenum.

The main imagistic methods of diagnosing liver and gallbladder pathologies are radiography and ultrasonography.

Radiography helps us determine volume changes that can appear at the liver. A method for objectively measuring the volume is by comparing the length of the liver to the thoracic vertebrae 11 (T11). The liver length is measured as the cranial border of the liver with the vena cava and the caudal border of the liver. In some breeds, like Pekinese or brachycephalic, the thoracic depth and width must also be taken in consideration. The normal gallbladder can't be evaluated on radiography.

Ultrasonography can evaluate the echogenicity of the liver parenchyma, the echostructure, the hepatic vessels, the gallbladder structure and the bile duct. The evaluation is made for diagnosing the normal liver, focal lesions, structure or architecture of the liver, vascular modification or the diameter of the bile duct.

MATERIALS AND METHODS

177 cases were examined during the interval 2018-2020 in the clinics of the Faculty of Veterinary Medicine from USV Iași. The main activity took part at the Radiology Laboratory and the Pathology Medical Service. During 30 september – 30 november 2019, the activity took part at the University "Federico II" from Naples, at the Radiology Laboratory. Ultrasonography was used as a golden standard in this study, while radiology was used at a small number of pacients.

The X-ray exam was made using the Intermedical Basic 4006 machine and the Examion X-CR SMART . The ultrasonographic exam was made using the GE Logiq V5 machine using a linear and a micro-convex probe.

RESULTS AND DISCUSIONS

From the total of 177 pacients examined using ultrasonography, 62 (35%) were diagnosed with pathologies of the gallbladder or biliary duct, 43 (25%) were diagnosed with liver pathologies this data being illustrated in figures 1 and 2. A correlation between biliary and hepatic pathologies couldn't be established, only 11% of the cases having both pathologies.

[1] Iasi University of Life Sciences (IULS)

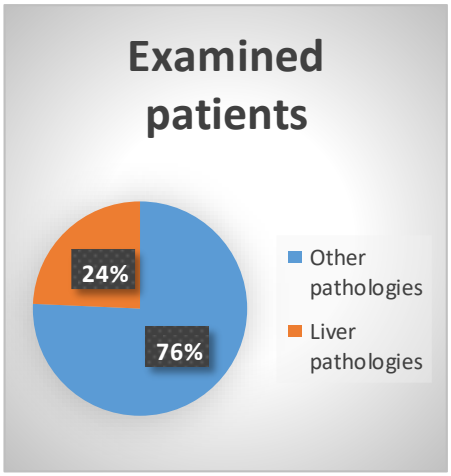

Figure 1. Clasification of pacients with liver pathologies

Figure 2. Clasification of pacients with gallbladder pathologies

There were a number of 26 (60%) females and 17 (40%) males with liver pathologies , while for gallbladder pathologies there were 33 (53%) males and 29 (47%) females . From this data we can observe that there is no correlation between liver and gallbladder pathologies and the sex of the animal.

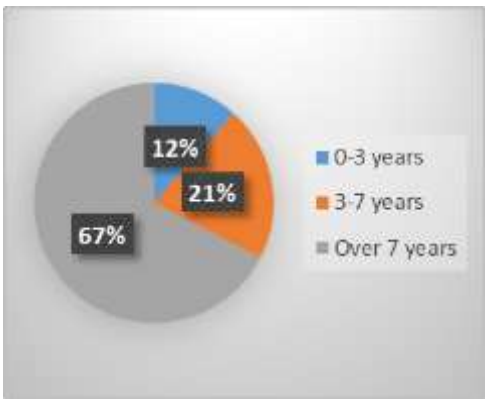

Figure 3. Age distribution of pacients with liver pathologies

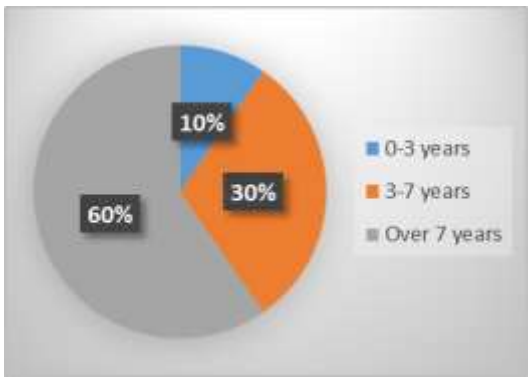

Figure 4. Age distribution of pacients with gallbladder pathologies

From figure 3 we can observe that geriatric pacients (over 7 years) are more predisposed to liver pathologies. This can be explained from the regeneration power of the liver and its functioning with a larger percentege of parenchima affected. Geriatric dogs have a slower metabolism and acumulate different lesions at the level of the parenchima during life. This two reasons can represente the explination of the liver pathologies at older dogs.

Pathologies between the age of 0-3 years are more rare, and they usually apear in case of genetic anomalies like porto-systemic shunts or different traumas.

From figure 4 we can observe the predisposition of gallbladder pathologies at the age category of over 7 years old. There were no genetic implications for the other age categories.

Mongrels in this study were represented by mix breeds that were under 20kg. We can observe from figure 5 that the biggest incidence of liver and gallbladder pathologies were at small breeds like bichon, caniche, pechinkez and some mongrels. This result can be explained by the bigger life expectancy of small breed races, meaning the higher chances of lesions to apear. Small breeds can have a life expectancy of 12-15 years while large breeds can reach 10 years.

The ultrasonographic modifications at the level o the liver were clasified into the following:

1. Dimension modifications such as hepatomegalia and microhepatia
2. Ecogenicity modifications such as hiperecogenicity in chornic hepatitis, fat liver, lymphoma or hipoecogenicity such as acute hepatitis.
3. Structural modifications that can be focal or diffuse
4. Vascular modifications such as vesel dillatation or porto-systemic shunts.

From figure 6 we can see that most of the modification were structural with a number of 20. This were represented by pathologies like: hepatic

nodules (figure 7), cysts and hepatic masses (figure 8).

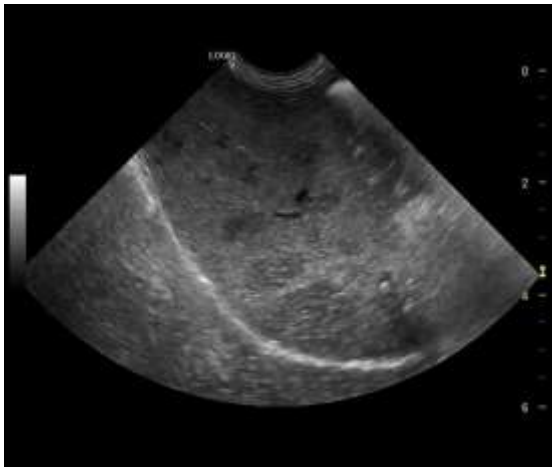

Figure 7. Multiple hyperechoic areas, difuse in the liver parenchim

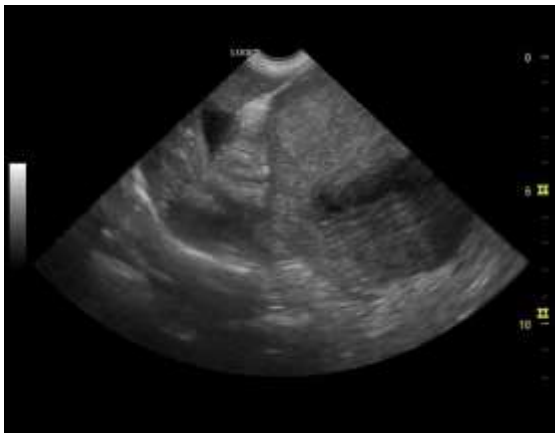

Figure 8. Retrohepatic mass with mixed ecogenicity

Dimension modification were represented by microhepatia and hepatomegalia. For a certain diagnosis, RX came to complete the ultrasonography exam.

The modifications at the level of the gallbladder where clasified into the following:

1. Wall modifications such as colecystitis
2. Bile content modifications such as sluge or sediment

The main modifications of the bile content are represented by sludge, that was usually found incidentaly, without any clinical signs, billiary calculi (fig 10) and mucocele (fig 11).

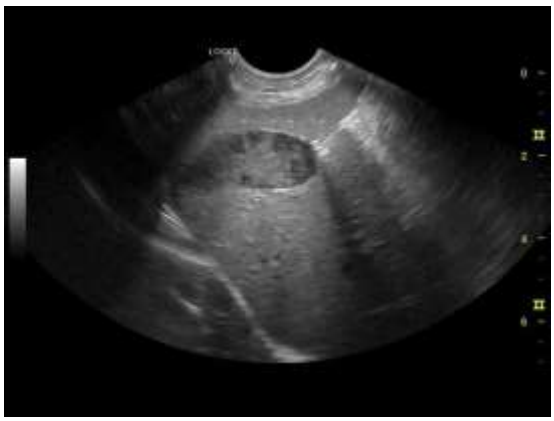

Fig 10. At the level of the gallbladder we can observe a high quantity of sediment shaped as a kiwi slice

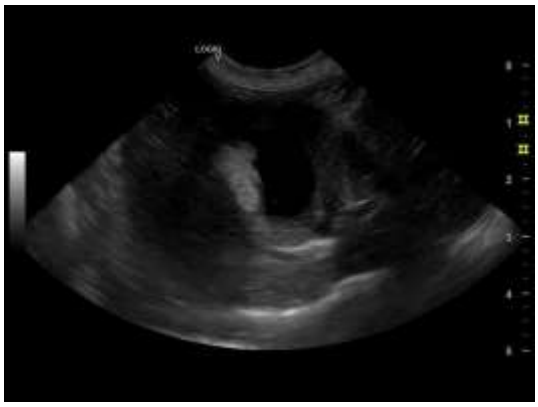

Fig 11. Hyperechoic sediment with acustic shadowing present

Modification of the biliary wall were repesented by colecystitis (fig 12) , angiocolystis and oedema (fig 13).

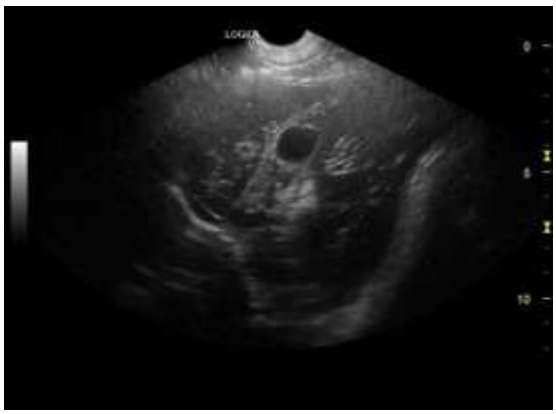

Fig 12. Thickening of the billiary walls and duct

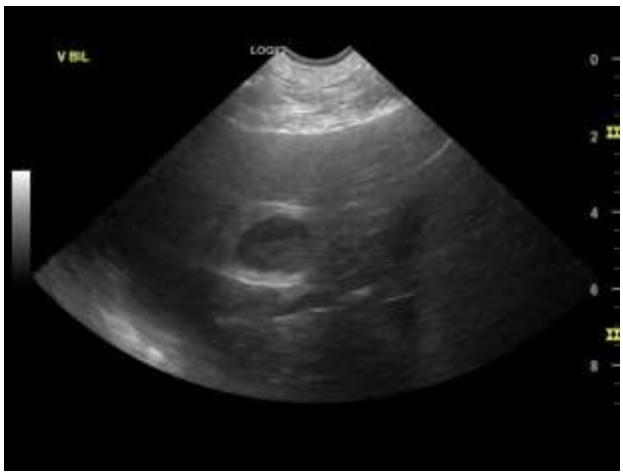

Fig 13. Thickening of the gallbladder wall with the apearence of 3 layers

CONCLUSIONS

In this study we have diagnosed using imagistics tehniques, completed in some cases by other paraclincal methods, liver pathologies like: liver masses, hepatic steatosis, hepatomegaly, microhepatia, distrofic changes at the level of the liver parenchima, porto-systemic shunts.

We can observe that the age that is more susceptible for liver pathologies is represented by the 3rd category (over 7 years) with a percentage of 67%, where the liver parenchima suffered different lesions during life and the regeneration power is reduced. In the category 0-3 years, we observed that the etiology is mostly genetic. There is no specificity for the sex of the animal.

We can also see that the prevalence of the liver pathologies is greater at the small breed dogs. An explination would be that their life expectance is higher.

In the case of gallbladder pathologies, we can observe that the dogs over 7 years (60%) had a higher incidence, followed by the pacients with the age between 3-7 years (30%) and lastly the pacients between 0-3 years (10%).

The small breed dogs were the most affected by this pathologies, the most representatives being Bichon.

Using the imagistic tehniques, mostly the ultrasonography , we have diagnosted the next pathologies of the gallbladder: billiary sludge, masses, colecistytis, angyocolitis, stones and wall oedema.

This research received no external funding.

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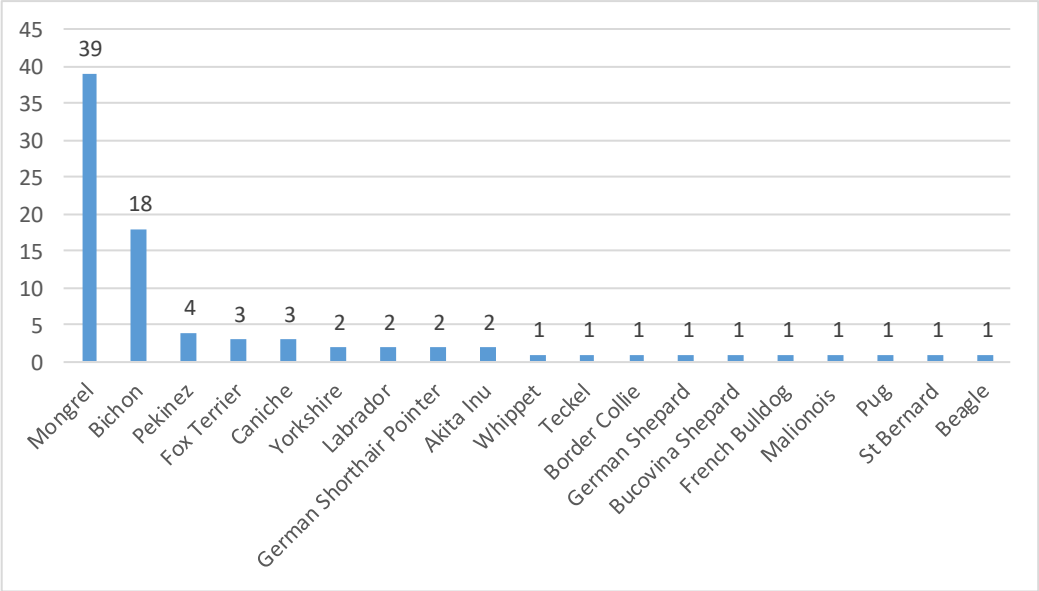


Figure 5. Distribution of breed in pacients with liver and gallbladder pathologies

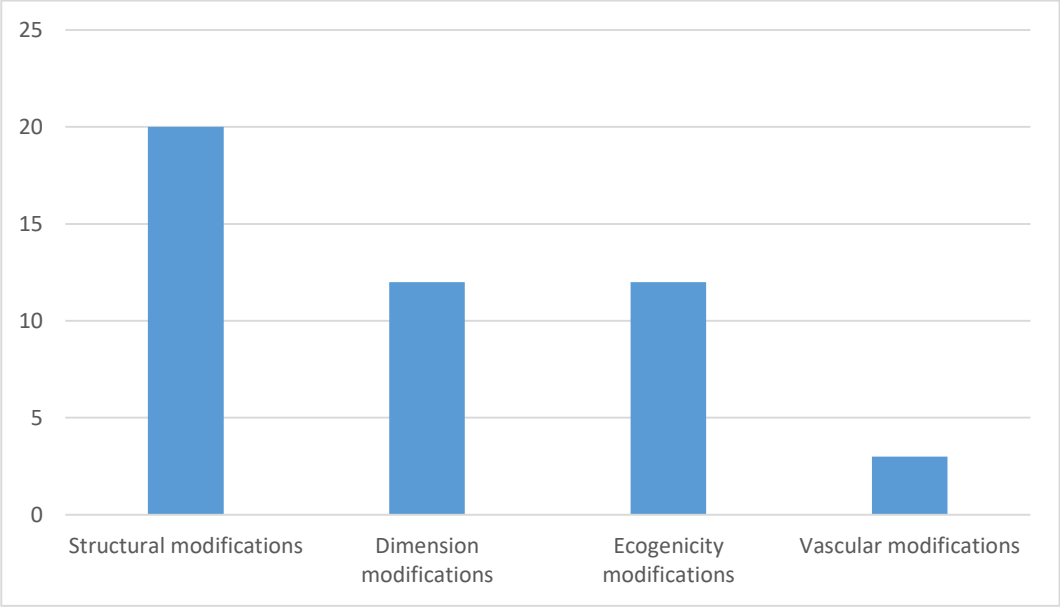


Figure 6. Ultrasonography modifications at the level of the liver parenchima

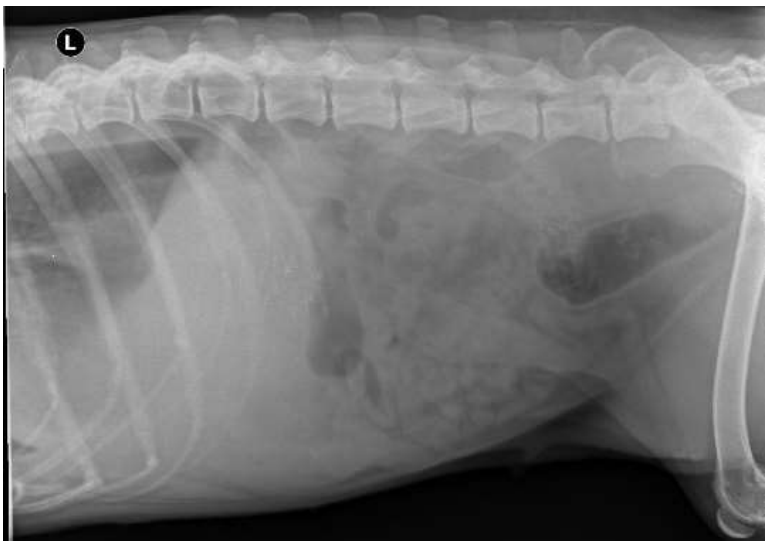
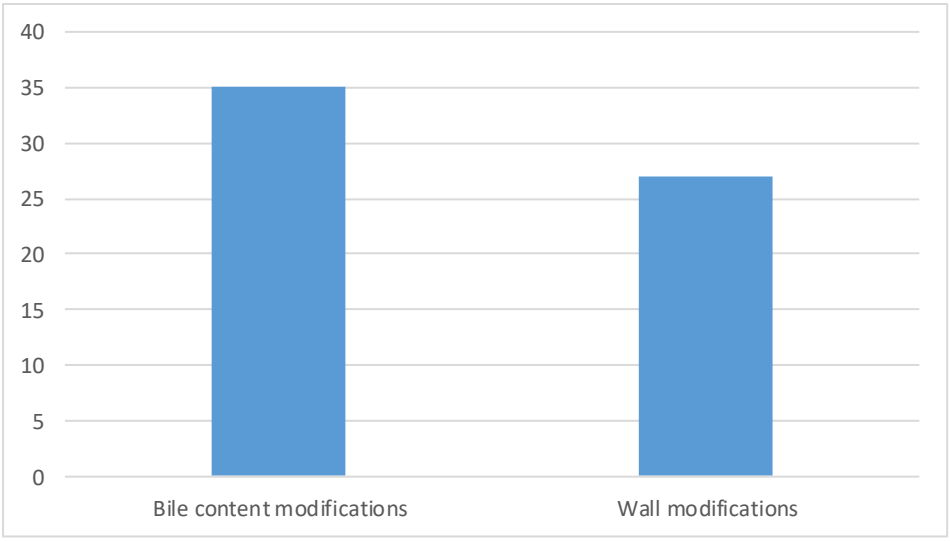


Figure 9. Hepatomegaly



CUTANEOUS PAPILLOMA IN CATTLE

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Abstract
Bovine papilloma virus (BPV) -2 is the most common on haired skin. A 4-year-old Fleckviech Simmental cattle, weighing 650 kg, with the presence of a formation on the upper eyelid of the right eye occupying approximately one third of it, was examined. The lesion was nodular, hyperkeratotic, cauliflower-like growths. Hematological abnormalities were lymphocytopenia, neutrophilia which indicates inflammation, infection, and hemoconcentration which indicates mild dehydration. Changes in liver enzymes combined with low blood sugar and ketone bodies in urine indicated ketosis status. Histological examination showed a hyperplastic epithelium covering an ordered proliferation of mesenchymal cells. Numerous large cells with blue-gray cytoplasm were visible within the hyperplastic epithelium. Corroborating the data of the clinical and paraclinical examinations, the diagnosis of cutaneous fibropapilloma was made.

Key words: bovine papillomaviruses, fibropapilloma, koilocytes

Bovine papillomaviruses (BPVs) infections are similar to human papillomaviruses (HPVs) when the PV gains access to a basal cell, probably due to microtrauma (Schiller J.T. et al, 2010) resulting in a productive infection of cutaneous or mucosal epithelium.

Papillomavirus infection of cattle causes mucocutaneous papillomas that develop on the haired skin, tongue, teats, penis, and vulva and upper alimentary papillomas that develop in the oral cavity, esophagus, and rumen. Mucocutaneous papillomas (warts) are more common in younger animals, and most cattle probably develop warts during their lives (Munday J.S., 2014).

MATERIAL AND METHOD

A 4-year-old Fleckviech Simmental cattle, weighing 650 kg, with the presence of a formation on the upper eyelid of the right eye occupying approximately one third of it, was examined at farm A from northeastern Romania.

Blood samples were obtained from the jugular vein using vacuum tubes containing EDTA for hematological examination of whole blood, and without anticoagulant for serum biochemical analysis.

Hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red and white blood cells count (RBC and WBC, respectively) analyses were done by an automatic analyzer (VetScan HM5®, Abaxis®, Germany) using reference levels for cattle (George J.W. *et al*, 2010).

Serum concentrations of alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, creatinine, urea nitrogen, glucose, total protein, albumin, globulin, amylase, sodium, potassium, calcium and phosphorus were determined in a biochemical automatic analyzer (VetScan VS2®, Abaxis®, Germany) using reference levels for cattle (Kaneko J.J. *et al*, 2008).

Fine needle aspiration biopsies (FNAB) and punch biopsy were performed from the skin lesion of the upper eyelid (*figure 1*). .

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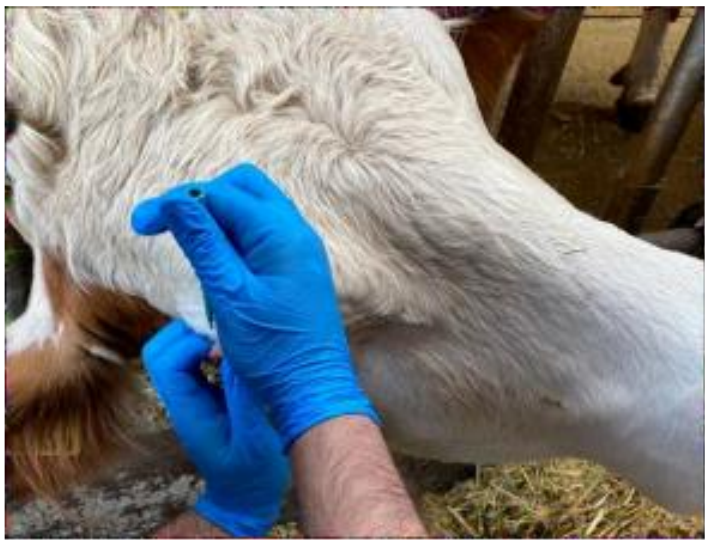

Figure 1 Punch biopsy of a cutaneous fibropapilloma in cattle

RESULTS AND DISCUSSIONS

Following clinical examination, our case was found to be clinically healthy, with no general changes in health. Examination of the skin confirmed the presence of a spherical formation, of hard consistency, located on the upper eyelid of the right eye, measuring 2cm / 1.5cm / 2cm, showing a slight increase in sensitivity of the area and a slight discomfort in blinking. The upper surface of the formation had a rough character and a gray color (*figure 2*)

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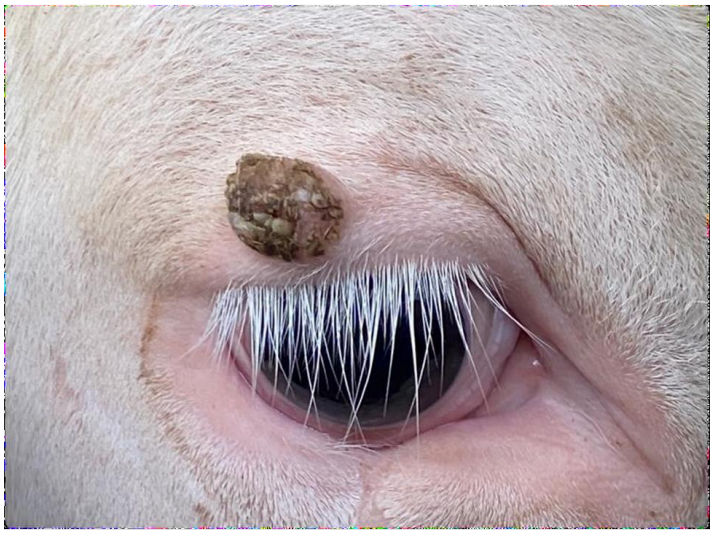

Figure 2 Fibropapilloma located on the upper eyelid of the right eye. The lesion is nodular, hyperkeratotic, cauliflower-like growths

Hematological abnormalities were lymphocytopenia (0.88 x10^9/L, normal 2.5-7.5), neutrophilia (9.03 x109/L, normal 1.8-6.3 x109/L) which indicates inflammation, infection, and haemoconcentration which indicates mild dehydration.

Serum biochemistry showed low albumin (2.2 g/dL, normal 3.03-3.55 g/dL) and high alkaline phosphatase values (199 UL, normal 27-107 U/L), indicating liver disease. Changes in liver enzymes combined with low blood sugar (40 mg/dL, normal 40-100mg/dL) and ketone bodies in urine indicated ketosis status.

Cytological examination following fine needle aspiration biopsie revealed the presence of erythrocytes and leukocytes and round epithelial cells, with an increased ratio of basophilic cytoplasm (*figure 3*).

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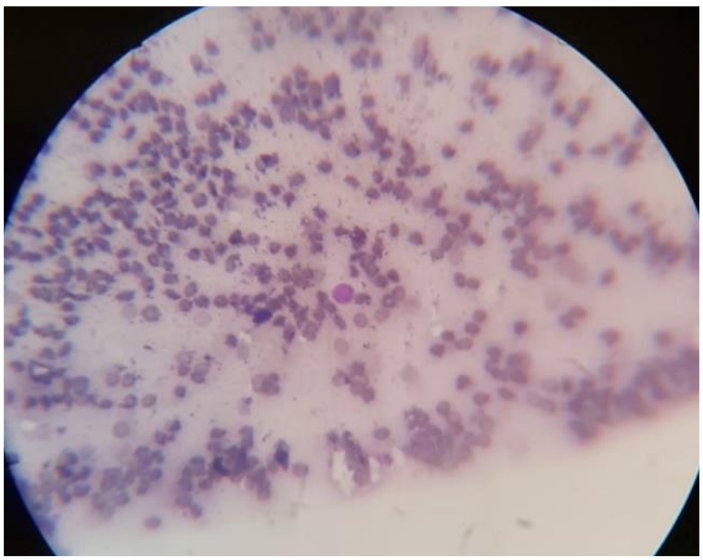

Figure 3 Erythrocytes, leukocytes and round epithelial cells with an increased ratio of basophilic cytoplasm from fine needle aspiration biopsies of a cutaneous fibropapilloma

Histological examination showed a hyperplastic epithelium covering an ordered proliferation of mesenchymal cells. The hyperplastic epithelium extends into the mesenchymal tissue. The area suggests a papillomaviral etiology, consisting of cells with clear, lightly colored cytoplasm in the superficial epidermis (Solcan C., 2011). Large keratinocytes are observed that contain dark, reduced nuclei, surrounded by a clear halo (koilocytes) often with agglomerated keratohyalin granules indicating fibropapilloma (*figure 4*).

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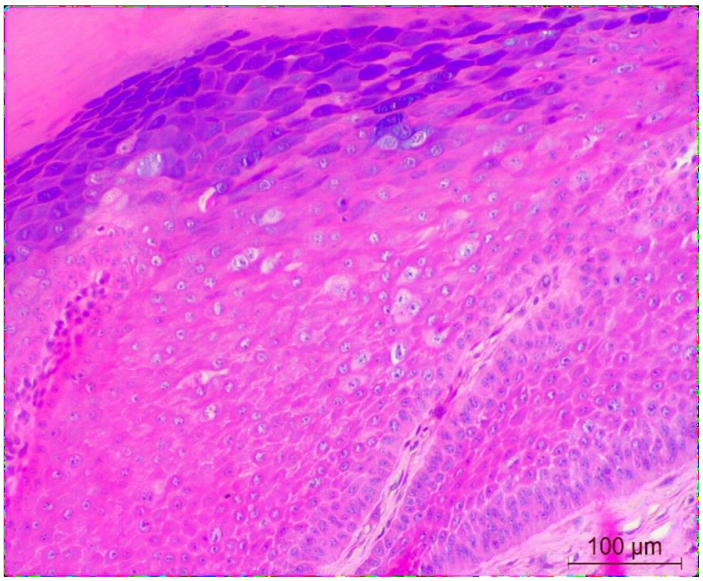

Figure 4 Cutaneous fibropapilloma in cattle. Numerous large cells with blue-gray cytoplasm are visible within the hyperplastic epithelium. Hematoxylin and eosin

Bovine mucocutaneous papillomas include squamous papillomas and fibropapillomas. Squamous papillomas have the same histological appearance as human papillomas. In contrast, fibropapillomas consist of a proliferation of mesenchymal cells covered by hyperplastic epithelium.

The mesenchymal cells are most densely packed close to the surface of the fibropapilloma (Jelinek F., Tachezy R., 2005). Fibropapillomas are generally considered to be caused by the delta-PVs BPV-1 and BPV-2. Current evidence suggests that most cattle are infected by BPV-2, and lymphocytes may provide a reservoir for infection (Stocco dos Santos R.C. *et al*, 1998; Roperto S. *et al*, 2011).

BPV-2 is able to infect and replicate in a wider range of tissues, including the transitional epithelium of the bladder (Roperto S., 2013) and the chorionic epithelium of the placenta (Roperto S. *et al*, 2012).

BPV-2 L1 protein production has also been reported in circulating lymphocytes, suggesting viral replication in nonepithelial cells.

Regarding the treatment, it is uncertain whether vaccination influences the regression of cutaneous papillomas in cattle. Injecting wart tissue harvested from the cow (autologous vaccination) has been widely used. In humans, most studies have not detected any therapeutic effect of vaccination after papilloma development (Vandepapeliere P., 2005).

CONCLUSIONS

Corroborating the data of the clinical and paraclinical examinations, the diagnosis of fibro papillomatosis was made. Given the increased likelihood of regression of the papillomatosis neoplastic formation and the complications that may occur after trying to treat this disease, it was decided that the application of a treatment would not provide satisfactory results, but her health continued to be monitored.

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BACTERIOLOGICAL AGENTS IN FARMED CYPRINIDS FROM THE PRUT RIVER BASIN

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Abstract

The current paper aims to outline what could be the most common bacterial agents that can have a pathogenic effect on farm cyprinids form the Prut river basin in order to reduce fish health risks and even prevent future disease outbreaks that are bacterial in nature. Two field works expeditions to Rompescaris farm -Podu-Iloaiei from Iasi county and to Dracșani fish farm from Piscicola-Botoșani county were undertaken in May 2021 in order to collect biological samples. The following fish species were harvested: carp (Cyprinus carpio), silver carp (Hypophthalmichthys molitrix) and mirror carp (Cyprinus carpio var. specularis) using net fishing and the only the specimens with visible lesions or the moribund ones were collected. The fish that were apparently healthy were released and the ones that were harvested underwent a clinical, parasitological and bacteriological investigation.

Key words: *Cyprinus carpio, Hypophthalmichthys molitrix, bacteriological, diseases*

Aquaculture is one of the fastest growing food-producing sectors, supplying approximately 40% of the world's fish food. Besides such benefit to the society, the industry does have its problems. There are occupational hazards and safety concerns in the aquaculture industry. Some practices have caused environmental degradation. Public perception to farmed fish is that they are ''cleaner'' than comparable wild fish. However, some farmed fish have much higher body burden of natural and man-made toxic substances, e.g., antibiotics, pesticides, and persistent organic pollutants, than wild fish (Cole W. David et al., 2009). Farmed fish as a result of overcrowding can be more susceptible to diseases that are caused by one or more species of pathogen or opportunistic bacteria.

Consumers may select farmed fish for meal as a healthier and safer alternative to wild fish because aquaculture is presumably located away from industries that generate contaminated air and water (Cole W. David et al., 2009) and because it is presumed that in a controlled environment the risk of disease is greatly reduced. That being said now more than ever when it is estimated that by 2030 aquaculture will produce 60% of all fish destined for human consumption (FAO 2020) we must pay more attention to aquaculture systems similar to those found in Moldova county in which farmed fish are kept at high population densities in close proximity with wild fish reservoirs because it is an ideal situation in which spreading of wild type pathogens form the wild fish to the farmed ones and vice versa (Kibenge et. al 2012) is greatly enhanced.

Several fish bacterial pathogens have zoonotic potential, but transmission is much more common from ingestion of raw or inadequately cooked or processed fish than from contact with fish.

The highest risk from contact with fish is direct wound inoculation (e.g., fish spine injury or bite wound). Most fish bacterial pathogens are also common in the environment and transmission is possible through water contact with mucosal surfaces, open wounds, or food, although this appears to be rare.

Disease is more likely with immune suppression (Hadfield A. Catherine and Clayton Leigh Ann, 2021).

That being said we hope that through this set of investigations we can better understand what

bacterial diseases are affecting farmed fish in the Prut river basin.

MATERIAL AND METHOD

Following the external and internal clinical examination, characteristic lesions of erythrodermatitis were observed. This disease is caused by the development of a single bacterial species or several species of conditionally pathogenic bacteria, as follows: *Aeromonas hydrophila, Aeromonas caviae, Aeromonas sobria, Pseudomonas aeruginosa, Schewanella putrefaciens or Plesiomonas shigelloides.*

The bacteriological examination was performed by inoculation of bacterial strains sampled from the injured tissues but also from the uninjured organs, on specific media (Brain Austin and Dawn Austin, 2007)

Inoculations were made from the spleen, kidneys, hepato-pancreas, gills, skin, from areas adjacent to the injured tissue and not from the center of the wound.

Using a sterile Pasteur pipette and a sterile loop, the organ was deeply pierced and pathological material was harvested, which was then deposited on the surface of a non-selective medium (TSA agar, nourishing agar, BHI agar,) and incubated at 25 ° C for 24 to 48 hours.

After performing the cultural examination, morphological identification was performed by Gram staining, and biochemical identification by inoculating the bacterial strains on biochemical media and the use of API diagnostic tests.

Detection of virulence factors, hemolysin production. Blood-based agar (Bio-Rad) supplemented with 5% sheep's blood was used to produce hemolysin and incubated at 37 ° C for 24 hours.

In order to identify the species within the genus Aeromonas, mass spectrometry (MALDI-TOF MS bioMérieux system) was used.

RESULTS AND DISCUSSIONS

After the incubation time expired, the Petri dishes were examined for bacterial colonies, and the morphology of the colonies was assessed using an optical microscope or a magnifying glass.

We took into account: the type of colonies, smooth (S) or rough (R), their diameter, regular or irregular edges, pigmentation, tendency to confluence and the convexity or concavity of bacterial colonies.

On triple sugar iron agar, we found a series of morphological characteristics depending on the bacterial strain

Hemolysin which is a major virulence factor in Aeromonas spp. Infection was observed in all isolates. Hemolysin activity was observed on blood agar plates (figure 1 and 2) which confirmed its presence.

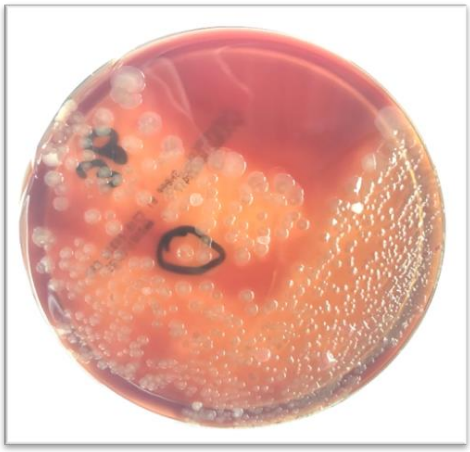

Figure 1. ***Aeromonas spp.*** **(Cultural aspect - blood agar)**

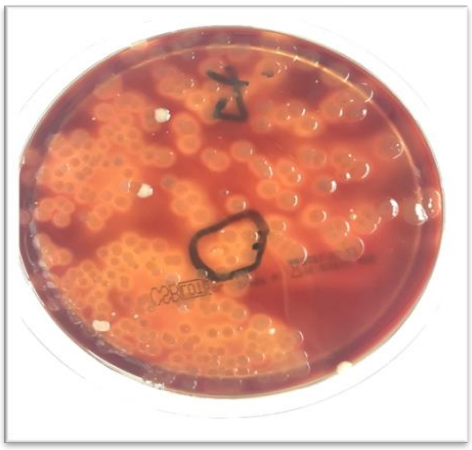

Figure 2. ***Aeromonas spp.*** **(Hemolysin activity)**

Testing the activity of bacterial strains against some sugars but also testing other characters is performed on TSI (triple sugar iron). It is a medium containing iron, 3 sugars in different concentrations (one monosaccharide = glucose and two disaccharides = lactose and sucrose) and a pH indicator (phenol red).

The medium poured into tubes was seeded using a loop with the bacterial strain to be tested in a column puncture or sloping grooves pattern.

The tubes were incubated at 25 and 37 ° C for 18, 24 and 48 hours, and the results were read.

Microorganisms that use glucose will induce an acidification of the medium until the sugar is depleted, causing the column of the medium to take on a yellow color. Bacteria that use lactose and / or sucrose will induce the same color change but in the slope of the tube - acidic PH.

If the bacteria don't use sucrose and lactose, then they degrade the protein (peptone) in the nitrogen-releasing substrate which will change the pH of the medium to alkaline and the color will shift to purple red.

Bacteria that ferment glucose with gas production will cause the accumulation of gas bubbles in the column, and bacteria that produce hydrogen sulfide, a compound that reduces iron sulfate from the environment to black iron sulfite, will cause blackening of the environment.

The results from testing the bacterial strains on TSI (triple sugar iron) are shown in table 1.

Table 1

Genus / species	Biochemical characters
Aeromonas hydrophila	Glu (+)/G(+) Lac (-) Suc (+)
Aeromonas caviae	Glu (+)/G(-) Lac (-) Suc (+)
Aeromonas sobria	Glu (+)/G(+)Lac (-)Suc (+)
Pseudomonas fluorescens	Glu (+)/G(+)Lac (-)Suc (-)
Schewanella putrefaciens	Glu (-)/G(-) Lac (-) H_2S (+)
Plesiomonas shigelloides	Glu (+)/G(-) Lac (+) H_2S (-)

Differentiation by growth at different incubation temperatures was also performed.

The pure bacterial cultures to be researched for 18-24 hours were seeded in tubes with non-differential medium (TSA) and incubated at 4°C, 25°C, 37°C and 41°C (table 2).

Biochemical testing with API tests was performed in order to highlight certain biochemical features and to identify the bacterial strain. The API test was chosen according to the species of bacteria that needed to be identified and the accuracy of the test.

The test was used according to the manufacturer's operating instructions and the identification was performed using the numerical profile on the test result sheet.

Table 2

Genus/ species	4°C	25°C	37°C	41°C
Aeromonas hydrophila	-	+	+	-
Aeromonas caviae	-	+	+	-
Aeromonas sobria	-	+	+/-	-
Pseudomonas fluorescens	+	+	-	-
Shewanella putrefaciens	-	+	-	-
Plesiomonas shigelloides	-	+	+	-

Antimicrobial susceptibility testing by diffusimetric method was also preformed and the interpretation consisted in appreciating the size of the zones of inhibition, induced by the antibiotic, area in which the microbial colonies are missing.

The diameter of the inhibition zones is measured in millimeters.

The results were marked with: "S" - sensitive, "R" - resistant bacteria and "MS" - moderately sensitive (table 3).

Table 3

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Antibiotic	The content in µg of the disc	Resistant area	Moderately sensitive area	Sensitive area
Oxytetracycline	30 µg	< 15 mm	15 -18 mm	≥ 18 mm
Enrofloxacin	5 µg	< 17 mm	18 – 21 mm	≥ 22 mm
Florfenicol	30 µg	≤ 16 mm	17 – 19 mm	≥ 20 mm
Flumequine	30 µg	< 21 mm	22 – 24 mm	≥ 25 mm
Erythromycin	15 µg	< 14 mm	14-17 mm	> 17 mm

During testing we got different results for each bacterial strain.

For *Aeromonas sobria* we found "S"-type colonies round with a diameter of 2-5 mm, regular, opaque, unpigmented edges (figure 3).

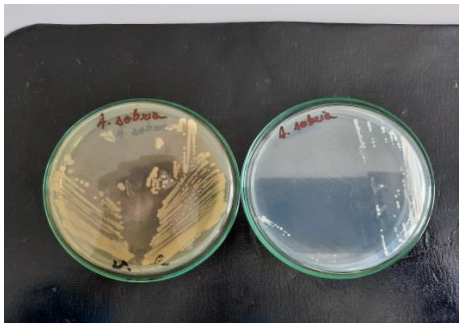

Figure 3. ***Aeromonas sobria*-cultural examination**

Antimicrobial susceptibility testing by diffusimetric method showed that *Aeromonas*

sobria was sensitive to florphenicol, doxycycline, enrofloxacin, trimethoprim and resistant to ampicillin, amoxicillin, erythromycin (figure 4).

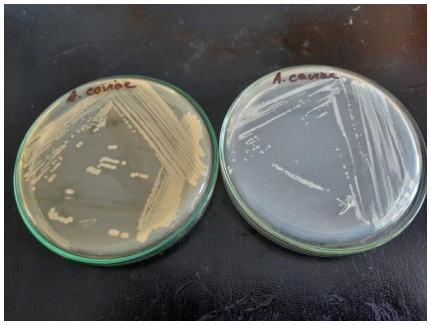

Figure 4. ***Aeromonas cavie*-cultural examination**

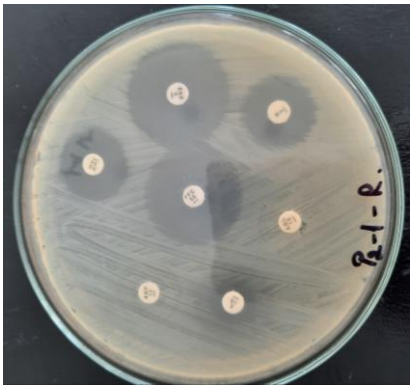

Figure 5. ***Aeromonas sobria*-susceptibility to antibiotics**

For *Aeromonas cavie* we found similar morphological characteristics (figure 5) and a slightly different susceptibility to antibiotics (figure 6).

Aeromonas cavie was sensitive to florphenicol, doxycycline, enrofloxacin, trimethoprim, erythromycin and resistant to ampicillin and amoxicycline.

CONCLUSIONS

Bacteria that are opportunistic and have the capability of causing a disease in fish can be found in water on a regular basis it is our aim to find out witch bacterial strains can cause problems more often and how to prevent or if necessary, treat these affections in fish populations.

Treatment using antibiotics can be efficient if done after a proper study or diagnostic but the actual administration of an active substance still poses some problems. The main obstacle is distributing the antibiotic in an even dose among the entire fish population, another problem is the resistance to antibiotics that can appear as a result of such treatments and the impact on the environment.

Most bacterial born diseases can be prevented in fish using a good management system but other pathogens such as parasites must also be managed very carefully because they can weaken the fish and make them more susceptible to bacterial strains that normally wouldn't cause any problems.

Through further studies we hope that in the near future we will better understand what bacterial strains found in the Prut river basin can cause diseases in fish and witch of them do so more often in order to reduce to a minimum the fish health risks in the area.

ACKNOWLEGMENTS

We would like to offer our thanks to the team of researches involved in the project "Team up for healthy fish in aquaculture systems of the Prut River basin 2SOFT / 1.2 / 47", researchers from the University of Life Sciences " Ion Ionescu de la Brad" from Iași (USV Iași) and the Institute of Zoology (IZ) of the Academy of Sciences in Chișinău (Republic of Moldova).

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CRUSTACEAN PARASITIC INVASIONS DIAGNOSED IN HYPOPHTHACHLMICHTHYS MOLITRIX (SLIVER CARP) IN THE DRACȘANI WATER ACCUMULATION

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Abstract

One of the objectives of the project Team up for healthy fish in the aquaculture system of the river Prut basin is to assess the health of fish populations; thus, the accumulation of Dracșani as a component part of the SC Piscicola Botoșani farm was studied. The parasitologically examined fish were of the silver carp (Hypophthalmichthys molitrix). Parasitic invasions were caused by Sinergasillus sp. and Lernaea sp. and the severity of the lesions were assessed.

Keywords: asian cyprinid, parasite, lesion

Fish, like other vertebrate species, react to environmental changes or the action of stressors through specific physiological mechanisms. When the functional capacity of these mechanisms is exceeded, a pathological condition can set in. According to several researchers, the disease state is the result of the close interaction between the pathogen, the host (fish) and the environment. Lately, in this relationship the environment is replaced by stress; the mathematical relation that explains this situation is in the form B = G + P + S, in which B - the disease, G - the host (fish), P - the pathogen, S - stressor.

Skin parasitic invasions can be caused by crustaceans and numerous sources in the literature point to infestations of this organ, in cyprinids, with parasites of the genus Lernaea; the lesions caused by this parasite are consistent with the intensity of the parasitism and the severity of the bacterial and fungal complications (Hastein, T., 2008).

The gills, in cyprinids, can also be parasitized by crustaceans ergasilidae, in this case by blood (*Hypophthalmichthys molitrix*), species of the genus *Sinergasilus*; In the same way, the lesions caused by this parasite are consistent with the intensity of the parasitism, but in the gills, the lesions can be more serious, through tissue damage, bleeding and inoculation of microbes and toxins.

MATHERIAL AND METHOD

Material and method. Dracșani Pond is located in the river basin of the Prut River, in the lower part of Sitna brook, right tributary of Jijia, on the upper part of Sulița commune, Botoșani county. the raised species are represented by the common carp, Asian cyprinids (silver carp, bighead carp, grasscarp), European catfish, salamander. The samples (fish specimens) were taken from the control fishing carried out in Drăcșani pond; specimens of different fish species were collected, but only silver carp samples of *Hypophthalmichthys molitrix* were studied for this study.

The clinical, anatomopathological (necropsy and morphopathological) examination was performed, as well as the parasitological examination according to the data from the specialized literature (Eissa A.E., 2016).

Clinical examination

The clinical examination began by conducting the clinical investigation and by direct examination of the fish; the latter was carried out on the shore during control fishing. Examination methods consisted of inspection and palpation. The inspection was initially applied to the fish gathered in the net, when approaching the shore (general appearance of the fish and swimming movements). The general inspection followed the assessment of the general conformation of the body, the lack of scales or the presence of some conformity anomalies. For each fish examined, the close inspection looked for general changes in the body or local (head, trunk, caudal peduncle, fins), as well as the presence of hypersecretion of mucus or macroscopic parasites on the body surface (skin and scales) and gills. (by the natural opening of the lids); In the case of blood specimens, at the examination of the body surface,

the presence of the parasite *Lernaea sp*. and lesions at the implantation site;

The gills were also inspected by opening the operculum, assessing the color, the integrity of the gill blades, the presence of mucus, bleeding and parasites. The presence of the crustacean *Sinergasilus sp*. and adjacent changes.

Anatomopathological examination

Each gill arch (after being detached by cutting at the ends) was placed on a glass slide and examined by hand magnifying glass. Whole fish were collected for the continuation of the parasitological examination in the laboratory, portions of gills with various modifications were collected and they were deposited in fixing solutions for the continuation of the examinations at the laboratory level.

During the examination, the necropsy examination was continued with the opening of the abdomen, the registration of the changes and the taking of samples; also samples were taken for bacteriological examination (these were not taken into account for this paper).

Parasitological examination

For the identification of ectoparasites (skin, fins, gills and eyeballs) the parasitological examination was performed either directly (by inspection, possibly with a magnifying glass) or by microscopic examination by native preparation, or by fixation, staining and microscopic examination of preparations. Scratches from the surface of the skin, fins and gills were also examined.

RESULTS AND DISCUSSIONS

Blood samples were examined parasitologically and anatomopathologically, identifying two types of parasitic invasions.

Parasitic invasion with Lernaea spp.

On examination of the skin and fins, the crustacean Lernaea spp. Was found with the presence of specific lesions at the implantation site. The crustacean was isolated and photographed, the anchor-like formation of the cephalic extremity, the fusiform body and the posterior extremity with the presence of ovarian sacs were highlighted. The intensity of the invasion had minimum values of 8 crustaceans / fish up to the maximum value of 51 parasites / fish. In specimens with intense infestation, the phenomenon of weakening and even emaciation was noticed due to the toxic and irritating action of the parasite.

Implants at the implantation site were examined by close inspection and with a magnifying glass, and were found to be clinically lesions of hemorrhagic-necrotic dermatitis.

Parasitic invasion with Sinergasilus spp.

Examination of the gills revealed the crustacean Sinergasilus spp. The intensity of the invasion had a minimum value of 1 crustacean/gill arch up to a maximum value of 3 crustaceans/gill arch. Fish were collected and portions of parasitic gills were introduced into fixative solutions for specific pathological examination. The parasitological examination continued, in the laboratory, with the laceration of the gill lamellae with spatulate needles to isolate the parasites and to perform microscopic examinations with a binocular magnifying glass, including with the preparation of permanent preparations and obtaining images.

Fig. 1 Silver carp, skin and fins invasion with *Lernaea sp*.

Fig. 2 Detail - crustacean *Lernaea sp*. attached to the skin

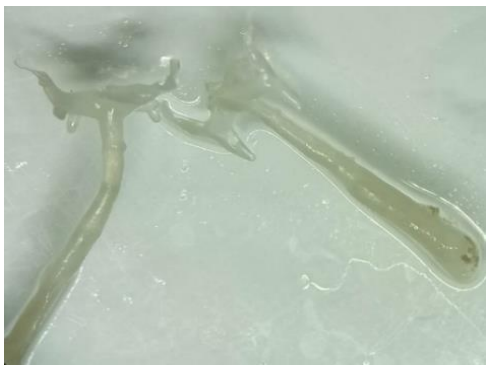

Fig.3 Two specimens of *Lernaea spp*. Detached from the skin, binocular magnifying glass image

Fig. 4 *Lernaea spp.* - detail of the anchor-type cephalic formation

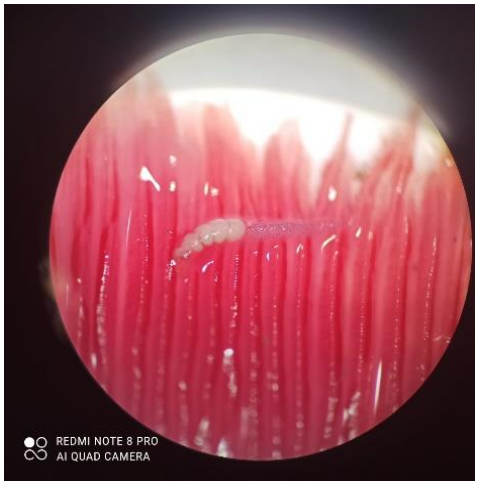

Fig. 5 *Sinergasilus sp.* – gills infestation, binocular magnifying glass image

Fig. 6 *Sinergasilus sp*., magn 100

Fig. 7 Sinergasilus spp. - image of cephalic antennae, permanently prepared, magn x 200

CONCLUSIONS

The studies carried out in Dracșani pond are at the beginning but show a medium severity parasitism with crustaceans at silver carp *Hypophthalmichthys molitrix*; the research will be continued in order to obtain complete data related to parasitological indicators (intensity, extensiveness) and control modalities.

ACKNOWLEGMENTS

We would like to thank the team of researches involved in the project "Team up for healthy fish in aquaculture systems of the Prut River basin 2SOFT / 1.2 / 47", researchers from the Institute of Zoology (IZ) of the Academy of Sciences in Chișinău (Republic of Moldova) and the University of Life Sciences " Ion Ionescu de la Brad" from Iași (USV Iași).

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ROLE OF A SELECTIVE PHOSPHODIESTETASE INHIBITOR IN TREATMENT OF INFLAMMATORY BOWEL DISEASE

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Abstract

This study was done on five groups of rats will be used (NC group), TNBS- untreated colitis, and three groups TNBS-treated colitis rats. Treatments will be oral Sulfasalazine (SS, 500), Roflumilast given by oral route (OR group, 5 mg/kg) or Roflumilast given by rectal route (RR group, 5 mg/kg). Treatments will be given daily for 15 days starting 48 hours after induction of colitis. Serum was collected for measurements of TNF-α, IL-2, IL-12 and cortisol which were done used ELISA kits. Tissues were collected for antioxidant enzymes (GSH-MDA-MPO) and for histopathological and immunohistochemically observations. All treatments significantly improved these changes. Sulfasalazine exerted the greatest effect followed by oral Roflumilast, and then by rectal Roflumilast. The aim of the current study is to evaluate the Roflumilast as a selective phosphodiesterase inhibitor in the treatment of colitis in rats.

Key words: Roflumilast, phosphodiestetase inhibitor, CD, rats

Inflammatory bowel disease (IBD) category is formed mainly of Crohn's disease (CD) and Ulcerative colitis. These diseases are of undefined etiology, most probably immunologically mediated, chronic, and progressive starting at young age and characterized by remissions, relapse and course protracted course (Ananthakrishnan, 2015). CD is a progressive disorder causing permanent intestinal injury and disability (Torres et al., 2017). More demonstration and understanding of IBD pathogenesis will provide appropriate management of the disproportionate and progressive inflammatory response to internal microbes in a genetically susceptible host (Geremia et al., 2014 and Cătană, et al., 2015).

The traditional management of IBD usually consists of the use of immunosuppressive besides anti-inflammatory drugs. For remission induction and maintenance, biological treatment could be used, and finally surgical treatment is introduced in case of failure to response to medical treatment (Gomollón et al., 2017). The anti-inflammatory effect of phosphodiesterase IV (PDE4) inhibitors is rather similar to that of corticosteroids with the advantage of non-interference with the hypothalamo-pituitary-adrenal axis. Roflumilast, a selective PDE-4 inhibitor with antiinflammatory and antifibrotic effects, is FDA-approved as an add-on treatment for COPD (Pauwels 2001, Hatzelmann et al., 2010). In animal models of colitis, when treatments are given before, during, or within one day after induction of colitis, any improvement could express a prophylactic and not a therapeutic effect because it may be simply due to an interference with induction of colitis. Thus, for the animal model to be relevant to human CD, it must be "chronic and immune- mediated" (Goyal et al., 2014). Also to predict clinical efficacy of a treatment in CD, it must be able to reverse "already-established" chronic colitis (Koboziev et al., 2011, Reardon 2016). In experimental models, trinitrobenzene sulfonic acid (TNBS) induces colitis with a Th-1 immune pattern (Randhawa et al., 2014).

Roflumilast is a very selective inhibitor of PDE4 used for severe COPD therapy. Its anti-inflammatory effect is mostly tolerable in comparison with previous inhibitors of PDE4. Such therapy proved dose-dependent enhancement of clinical scoring, length of colon, and production of TNF- α in the colonic mucosa (Rieder et al., 2013). In experimental models, it lowers inflammatory response in different disease. E.g. in COPD associated with bacteria, therapy with Roflumilast decreases lung infiltration by leukocyte, suppress inflammation, and liver damage was prevented. Moreover, it decreased pro-inflammatory cytokines levels in the serum of COPD patients (Feng et al., 2017).

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MATERIAL AND METHOD

Five groups of rats (n=8) will be used (NC group), TNBS-untreated colitis (Chronic colitis was induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), and three groups TNBS-treated colitic rats. Treatments will be Sulfasalazine (SS, 500), Roflumilast given by oral route (RO group, 5 mg/kg) and Roflumilast given by rectal route (RR group, 5 mg/kg). Treatments will be given daily for 15 days starting 48 hours after induction of colitis. Serum was collected for measurements of TNF-α, IL-2 and IL-6 which were done used ELISA kits. Tissues were collected for antioxidant enzymes (GSH- MDA-MPO) and for histopathological and immunohistochemical observations.

Statistical test:

Data were expressed as means ± SEM and analyzed using SPSS version 22. One-way ANOVA and Tukey's post hoc tests were used to test differences among groups. P < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Colonic measurements

The TNBS-colitic rats showed significant increases of the contents of MPO (a marker for neutrophil infiltration), and MDA and a significant decrease of GSH level in the colon homogenate. All treatments significantly reversed these TNBS-induced changes with significant differences in-between. Sulfasalazine exerted the greatest changes while oral roflumilast caused more changes than rectal roflumilast. (Tables 1- 3 and Figures 1-3).

Table 1

Effects of Roflumilast on colonic levels of MDA in TNBS-induced colitis (n==8)

Groups	Mean ±SEM
Control healthy	19.31 ± 0.66
TNBS	81.40 ± 2.13
Sulfasalazine	28.93 ± 0.50 [d]
Oral Roflumilast	36.68 ± 1.98 [d, e, f]
Rectal Roflumilast	47.04 ± 1.98 [d]

Data are expressed as mean ± SEM. d: P < 0.001 OR & RR vs. NC and All treatments vs. PC, e: P < 0.01 OR vs. RR (= 0.001), f:P < 0.05 OR vs. SS (= 0.014).

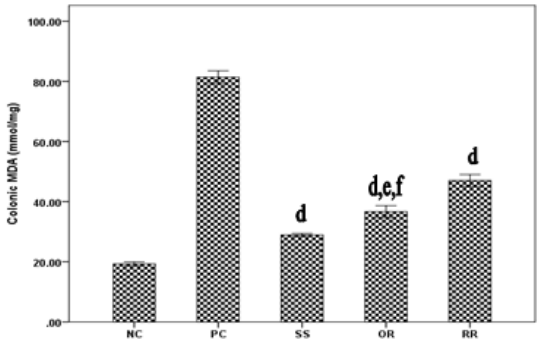

Figure 1. **The MDA content in different rats groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Table 2

Effects of roflumilast on colonic levels of GSH in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	19.60 ± 0.79
TNBS	6.86 ± 0.22
Sulfasalazine	14.73 ± 0.49 [g]
Oral Roflumilast	11.94 ± 0.32 [g, h]
Rectal Roflumilast	9.24 ± 0.28 [g, h]

Data are expressed as mean ± SEM. g : P < 0.001 All treatments vs. NC and SS & OR vs. PC, h : P < 0.01 RR vs. PC (= 0.008), OR vs. SS & RR (= 0.001, 0.002).

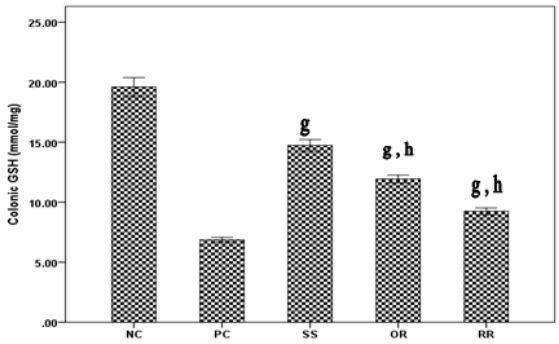

Figure 2. **The GSH content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Table 3

Effects of roflumilast on colonic levels of MPO in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	11.29 ± 0.85
TNBS	50.70 ± 1.05
Sulfasalazine	18.38 ± 0.87 [a]
Oral Roflumilast	23.89 ± 0.92 [a, b, c]
Rectal Roflumilast	28.17 ± 1.00 [a]

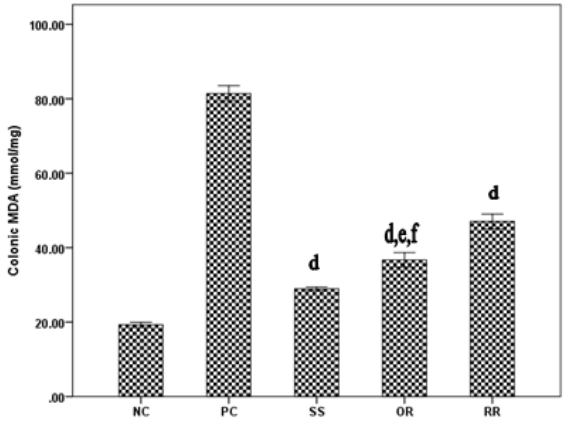

Figure 3. **The MPO content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Serum measurements:

The TNBS colitic rats showed significant increases of the serum TNF-α, IL-2, and IL-6. All treatments significantly reversed these TNBS-induced changes with significant differences in-between. Sulfasalazine exerted the greatest reductions while oral roflumilast caused more reductions than rectal roflumilast (Tables 4- 6 and Figures 4-6).

Table 4

Effects of roflumilast on serum levels of TNF-α in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	17.04±0.67
TNBS	91.18 ± 4.14
Sulfasalazine	28.93 ± 0.5 [a, b]
Oral Roflumilast	38.87 ± 2.33 [a, c]
Rectal Roflumilast	79.37 ± 1.88 [a, b]

Data are expressed as mean ± SEM. a : P < 0.001 Oral roflumilast (OR) & Rectal roflumilast (RR) vs. Normal control (NC) and Sulfasalazine (SS) & OR vs. Positive control (PC), and RR vs. SS & OR. b : P < 0.01 SS vs. NC (= 0.008) and RR vs. PC (=0.008), c : P < 0.05 OR vs. SS (= 0.034).

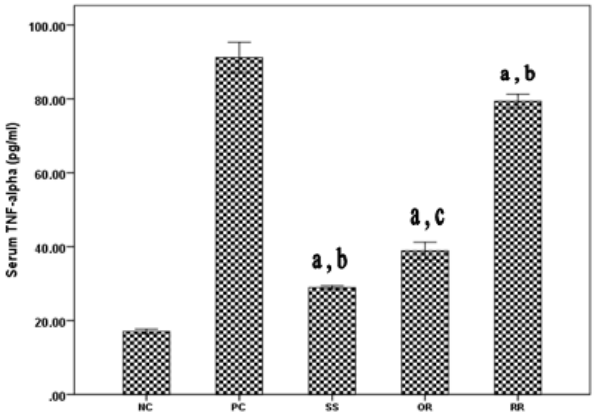

Figure 4. **The TNF content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Table 5

Effects of roflumilast on serum levels of IL-2 in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	196.10 ± 3.19
TNBS	487.00 ± 9.36
Sulfasalazine	257.97 ± 7.72 [d]
Oral Roflumilast	302.75 ± 11.32 [d, e, f]
Rectal Roflumilast	363.87 ± 12.73 [d]

Data are expressed as mean ± SEM. d : P < 0.001 All treatments vs. NC & PC, e : P < 0.01 OR vs. RR (= 0.001), f : P < 0.05 OR vs. SS (= 0.016).

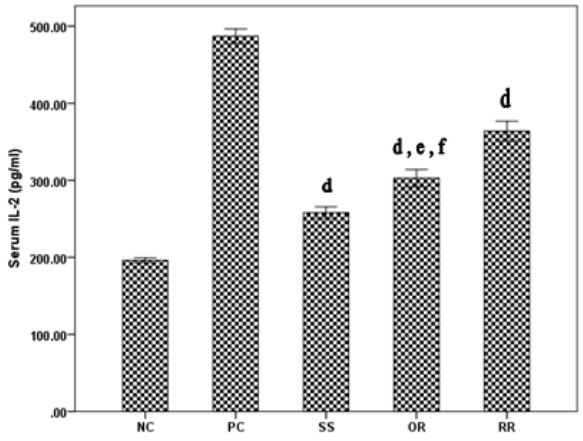

Figure 5. **The IL2 content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test**

Table 6

Effects of roflumilast on serum levels of IL-6 in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	10.57 ± 0.42
TNBS	47.04 ± 1.98
Sulfasalazine	16.14 ± 1.13 [g, i]
Oral Roflumilast	21.85 ± 1.00 [g, h]
Rectal Roflumilast	28.93 ± 0.50 [g]

Data are expressed as mean ± SEM. g : P < 0.001 OR & RR vs. NC and All treatments vs. PC, h : P < 0.01 OR vs. RR (= 0.001), i : P < 0.05 SS vs. NC & OR (= 0.013, 0.010).

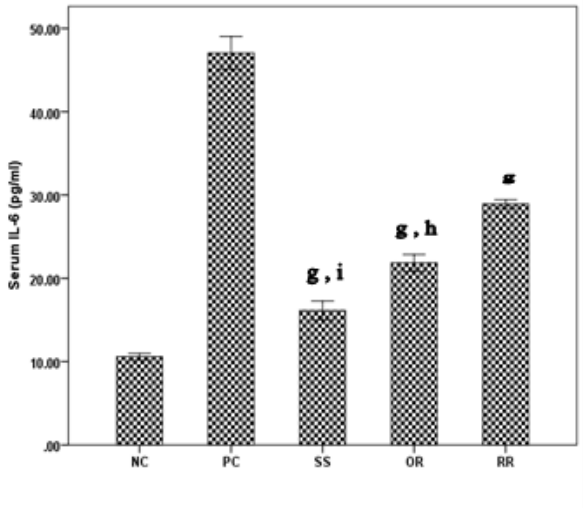

Figure 6. **The IL6 content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Histopathological examination:

In the colonic sections stained with H&E, the TNBS-colitic rats revealed destroyed glands, marked inflammatory infiltration, focal necrosis of mucosa and submucosa, loss of lining epithelium, and diffuse submucosal edema (Figure 7). For Masson trichrome, the TNBS group showed numerous congested blood vessels in the propria submucosa with edema around the CT fibers and blood vessels. Accumulation of lymphocytes was located in the propria submucosa, and some large nodules were located in the mucosa-submucosa (Figure 8). For PAS, the TNBS group showed numerous goblet cells with positive PAS reaction prominent between the lining epithelium of the crypts. Some crypts of Lieberkühn showed ulceration and accumulation of leucocytes (Figure 9). For the three stains, all treatments reversed these TNBS-induced changes with varying degrees. Sulfasalazine exerted the greatest improvement and oral roflumilast caused more improvement than rectal roflumilast.

Immunohistochemical results:

In the colonic sections stained for Ki 67, the TNBS-colitic rats revealed very faint positive reaction (Figure 10) while for immunohistochemical staining for TNF-α, the TNBS group showed strong positive reaction (Figure 11). For the two stains, all treatments reversed these TNBS-induced changes with varying degrees. Sulfasalazine exerted the greatest improvement and oral roflumilast caused more improvement than rectal roflumilast.

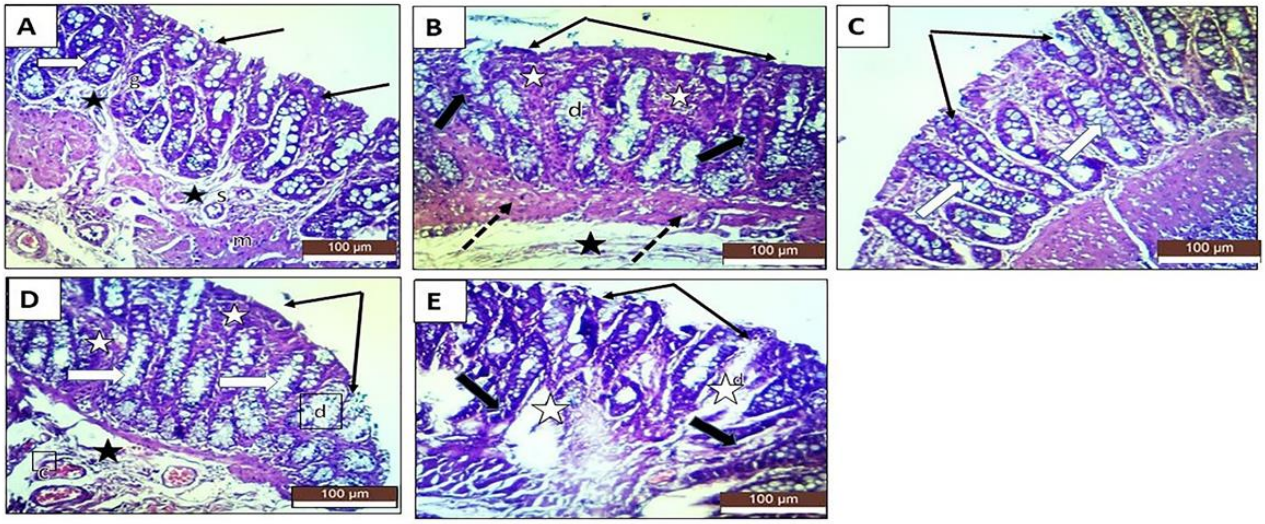

Figure 7. **Microphotographs of colon sections showing effects of oral and rectal roflumilast (H&E, X20). (A) Normal control group showing normal colonic structure with tunica submucosa consisting of loose CT rich in blood vessels and lymph vessels (s) and tunica musculosa consisting of inner circular and outer longitudinal smooth muscle fibers (m). (B) Positive control group showing destruction in the glands (d) and marked inflammatory infiltration. (C) Sulfasalazine group showing moderate to marked improvement with few desquamated cells especially the colonocytes (d) and minimal inflammatory infiltrate. (D) Oral roflumilast group showing moderate improvement with thickening of the colonic wall with patches of fibrosis (f) and epithelial desquamation (d). (E) Rectal roflumilast group showing mild improvement with desquamated colonic cells (d) especially the cells lining the crypts and with numerous congested blood vessels (c) in the propria submucosa.**

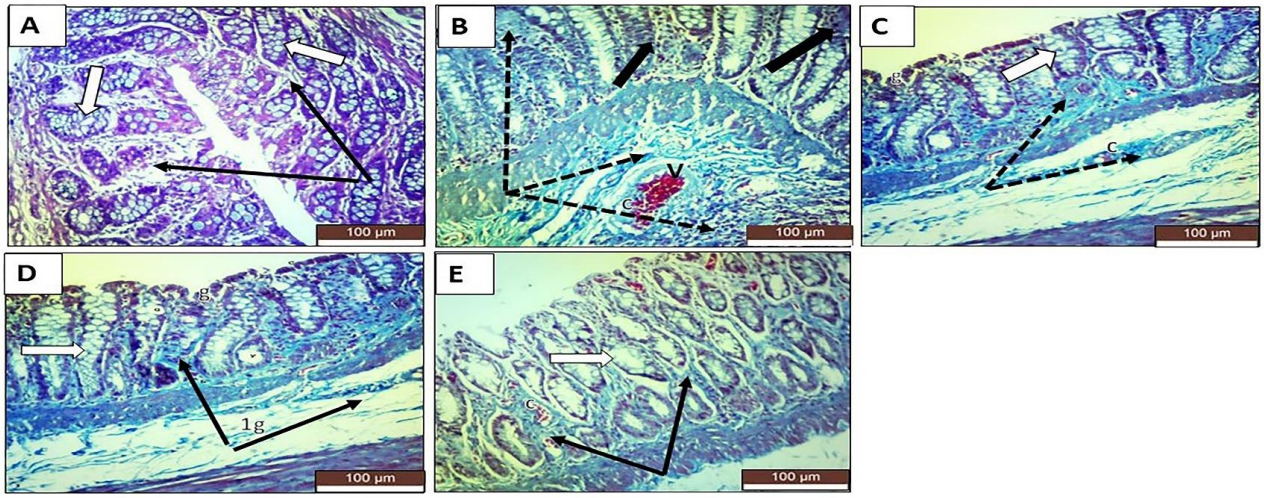

Figure 8. **Microphotographs of colon sections showing effects of oral and rectal roflumilast (Masson trichrome, X20). (A) Normal control group showing normal colonic collagen fibers (lg) and diffuse lymphocytes in the propria submucosa and numerous goblet cells (g). (B) Positive control group showing numerous congested blood vessels in the propria submucosa with edema around the CT fibers and blood vessels (c). Accumulation of lymphocytes was located in the propria submucosa, and some large nodules were located in the mucosa-submucosa (l). (C) Sulfasalazine group showing some congested blood vessels (c) in the propria submucosa and diffuse lymphocytes and numerous goblet cells (g). (D) Oral roflumilast group showing numerous congested blood vessels (c) between the crypts of Lieberkühn (intestinal glands). (E) Rectal roflumilast group showing some ulcerated crypts of Lieberkühn (u) with accumulation of leucocytes. 284x144mm (300 x 300 DPI)**

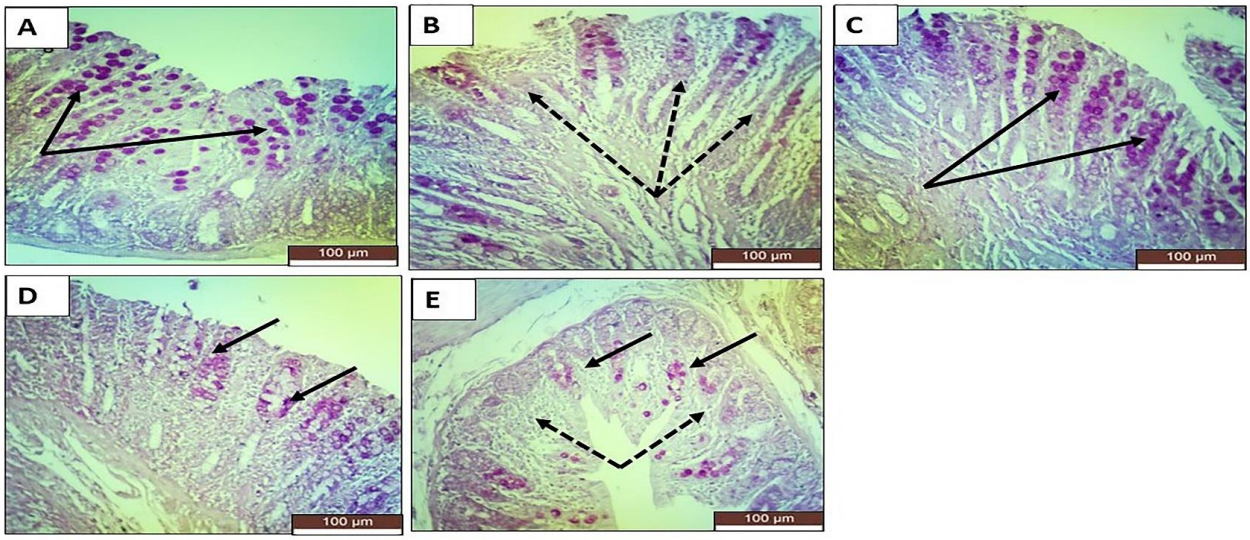

Figure 9. **Microphotographs of colon sections showing effects of oral and rectal roflumilast (PAS, X 20). (A) Normal control group showing numerous goblet cells with positive PAS reaction between the enterocytes (g). The propria submucosa CT showed very faint PAS positive fibers. (B) Positive control group showing numerous goblet cells with positive PAS reaction (g) prominent between the lining epithelium of the crypts. Some crypts of Lieberkühn showed ulceration and accumulation of leucocytes. (C) Sulfasalazine group showing PAS-positive goblet cells while the CT of the propria submucosa showed faint positive PAS reaction (g). (D) Oral roflumilast group showing goblet (g) cells with faint positive PAS reaction. (E) Rectal roflumilast group showing PAS-positive goblet cells of the crypt lining. 261x159mm (300 x 300 DPI)**

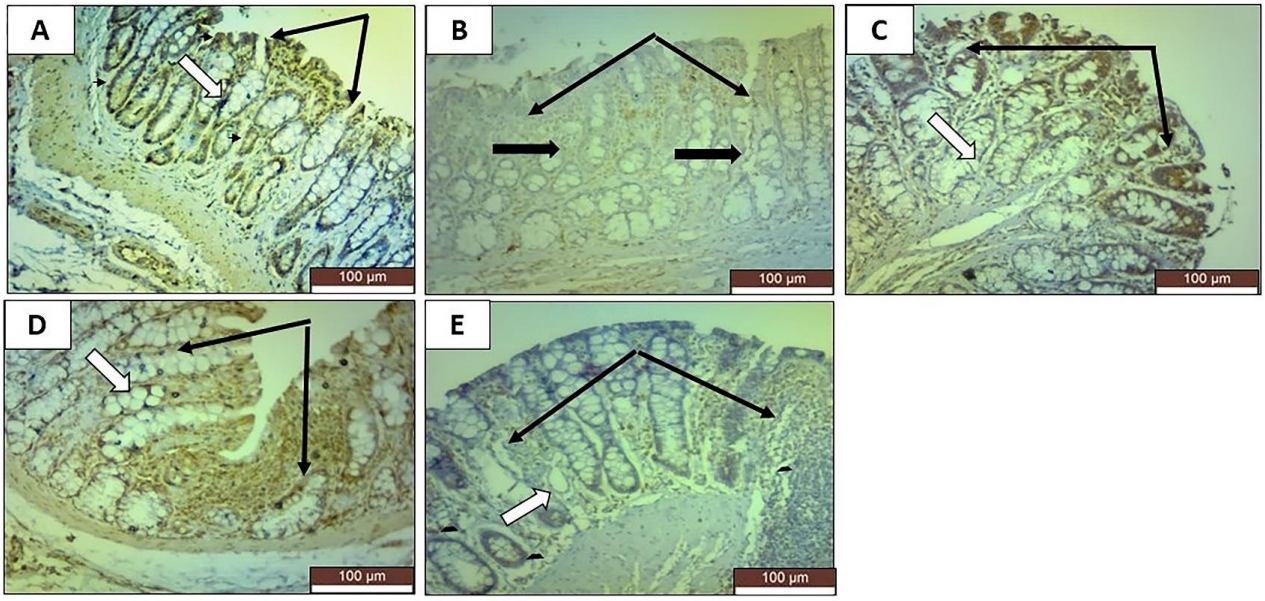

Figure 10. **Immunostaining for Ki-67 of colonocytes and crypt cells showing effects of oral and rectal roflumilast. (A) Normal control group showing positive reaction. (B) Positive control group showing very faint positive reaction. (C) Sulfasalazine group showing faint positive reaction. (D) Oral roflumilast group showing faint positive reaction. (E) Rectal roflumilast group showing very faint positive reaction (20X). 284x144mm (300 x 300 DPI)**

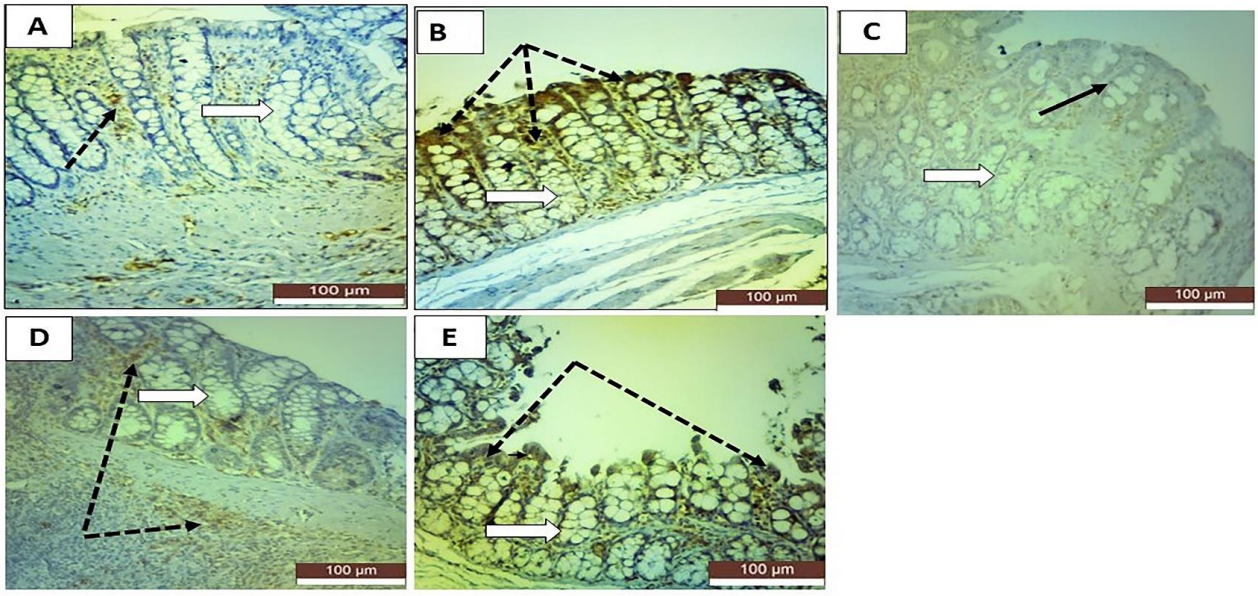

Figure 11. **Immunostaining for TNF-α of colonocytes and crypt cells showing effects of oral and rectal roflumilast. (A) Normal control group showing negative reaction. (B) Positive control group showing positive reaction. (C) Sulfasalazine group showing very faint positive reaction. (D) Oral roflumilast group showing moderate positive reaction. (E) Rectal roflumilast group showing strong positive reaction (20X). 270x161mm (300 x 300 DPI)**

The phosphodiesterase IV (PDE4) is an intracellular enzyme which increases production of the proinflammatory mediators and decreases production of the anti-inflammatory mediators, and thus it is implicated in pathogenesis of many inflammatory diseases. PDE4 inactivates cyclic adenosine monophosphate (cAMP) and is the main PDE isoenzyme in the mononuclear inflammatory cells, the main source of production of TNF-α. It was reported that elevation of TNF-α plays a crucial role in the pathogenesis of inflammatory bowel disease (IBD). IL-2 is another common cellular inflammatory factor. In inflammatory conditions, levels of TNF-α and IL-2 rapidly increase and thus activate WBCs, promote the migration of inflammatory cells, and expand the inflammatory response (Oh et al., 2017). Inhibition of PDE4 is associated with a broad anti-inflammatory activity including suppression of TNF-α. Thus, specific inhibition of PDE4 could be effective in treatment of several chronic inflammatory disorders (Banner and Trevethick, 2004, Li et al., 2018, Loher et al., 2003). Inhibition of PDE4 might provide a novel approach in the prevention and treatment of IBD (Videla et al., 2006). Unfortunately, clinical use of PDE4 inhibitors such as rolipram was limited by their adverse effects, but interestingly, roflumilast, the first-licensed member in this class, is a highly selective PDE4 inhibitor and is clinically effective at a relatively low dose compared with other PDE4 inhibitors. It improved episodic memory in subjects with minimal cognitive impairment at a non-emetic dose with plasma levels of about five times lower than the approved dose for COPD treatment. Use of these low doses minimizes the typical side effects of the PDE 4 inhibitors such as vomiting (Sugin et al., 2020). Roflumilast showed a more potent anti-inflammatory activity in both animals and humans and was more well-tolerated than the early PDE4 inhibitors like rolipram and cilomilast (Bundschuh et al., 2001).

The results of the current study agree with a previous study which revealed that in mice with dextran sulphate sodium (DSS)-induced colitis, oral roflumilast (1 or 5 mg/kg/d) dose-dependently improved the disease clinical score (weight loss, stool consistency and bleeding), colon length, and colonic TNF-α production. However, it did not improve the histologic score (Rieder et al., 2013). In addition, roflumilast showed potential anti-inflammatory effects in DSS-induced ulcerative colitis in male Wistar rats. Colitis was determined by assessing colon length, weight loss, histologic colon score, and measuring the concentrations of TNF-α, nitric oxide, cAMP, MPO activity and inducible nitric oxide synthase (iNOS) gene expression in colonic tissue. Roflumilast (5 mg/kg) attenuated the severity of colitis as evidenced by increased colon length, improved body weight loss, and improved colon histologic score compared to the DSS group. It also decreased colon concentrations of TNF-α, NO and MPO activity and down-regulated the iNOS gene expression. The results of roflumilast were comparable to those exerted by sulfasalazine (El-Ashmawy et al.,

2018). The colon length is an indirect measure for the severity of colonic inflammation (Loher et al., 2003). In agreement with our results, roflumilast was reported to partially reverse the TNBS-induced reduction in colon length at 1 and 5 mg/kg/day and to decrease the elevated colonic TNF-α concentration (Rieder et al., 2013). Moreover, in sepsis-induced liver damage in mice, roflumilast inhibited the expression of TNF-α, and IL-6 (Feng et al., 2017).

In the current study, we examined the effects of rectal roflumilast on TNBS-induced CD in a trial to prove that local roflumilast through rectal application could help in treatment of CD. Our results showed that rectal roflumilast reversed the TNBS-induced colitic changes indicating local anti-inflammatory effects. A previous study reported that oral roflumilast has local anti-inflammatory effects. In that study, colitis was induced in rats and the local effects of oral roflumilast at sites of inflammation were examined. It was found that treatment with oral roflumilast dose-dependently ameliorated the clinical score of colitis, led to a reduced shortening of the colon length, and decreased local expression of TNFα in colonic tissue, but this improvement was not associated with lowering the histologic score (Rieder et al., 2013). In addition, in a clinical trial, roflumilast cream applied topically once daily to affected areas of psoriasis was superior to vehicle cream in causing to an almost clear state at six weeks. Thus, it seems that topical roflumilast has the potential to help existing therapies in many inflammatory skin diseases (Lebwohl et al., 2020). Moreover, it is known that activation of adenylate cyclase by prostaglandin E2 or prostacyclin may exert a synergistic effect with PDE inhibition to enhance cAMP and reduce inflammatory cellular effects (Sinha et al., 1995). The inflamed mucosa in IBD patients has elevated levels of prostaglandin E2 and prostacyclin and therefore, administration of specific PDE inhibitors might lead to the strongest local effects in the gut. Consequently, although the extent of improvement with rectal roflumilast is less than that with oral roflumilast, but this improvement could suggest a potential role of rectal roflumilast as an add-on therapy for CD.

In the current study, roflumilast improved the histopathological and immunohistochemical changes induced by TNBS in rats. This agrees with a previous study which showed a close relation between crypt lesions and clinical activity of colitis (Cooper et al., 1993), but contradicts with another study which reported non-significant change on the histologic score with roflumilast (Rieder et al., 2013). The antigen KI-67 is a nuclear protein that is considered a marker of cellular proliferation. KI-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent in resting (quiescent) cells (G0) (Cuylen et al., 2016). In DSS-induced UC, epithelial apoptosis increased approximately 5-fold and the mitotic cells decreased about half-fold as compared to the controls. KI-67 immunohistochemistry showed that cells with cell cycle arrest at the G0 stage in the crypt increased approximately 2-fold as compared to the controls indicating decreased proliferation. This might lead to a breakdown of the epithelial barrier function, and thus facilitate the mucosal invasion of intraluminal microorganisms in DSS-induced colitis (Araki et al., 2010).

The colon of the second group (TNBS) showed desquamation of the colonocytes and destruction of some crypts, congested blood vessels were noticed with diffuse odema in the propria and submucosa, the goblet cells were increased and the crypts of liberkhum showed ulceration and accumulation of leucocytes.

These findings were supported by the results of (Cosnes, 2011, Danes, 2011 and De souza, 2016) which showed lesions of different size are simultaneously present. The mucosa may appear normal or may show multiple small (1–2 mm in size) punctiform, rounded nodules or superficial erosions known as ‘aphthoid lesions’. Over a period of time, the erosions become confluent and give rise to larger longitudinal ulcers, known as serpiginous ulcers.

the results of (Feller, 2007, Feng, 2017, Gajendran, 2018, Germia and Jewell, 2014 and Germia et al.,2012) showed ulcers at the base of crypts with neutrophils streaming into the bowel lumen, which leads in a later phase to mountain peak ulcers, villous abnormalities, and damage of small capillaries (including capillary thrombi) with subsequent loss of surface epithelial cells, these results were similar to our finding typically in group one (control positive group)

The lymphocytes were accumulated in the propria submucosa in the form of lymphoid nodules which obsecured the underlying structure and may lead to obstruction of the lymph vessels. These results were supported by the finding of (Van Kruiningen et al., 2014) which recorded a dense network of lymphocytes, histocytes, and macrophages within the lymphatic system results in the obstruction of regional lymphatics. This complex structure was observed in all layers of the intestinal wall in CD. Coincidently, transmural inflammation, multiple lymphoid aggregations in the submucosa, and beaded changes of the serosa occurred only where the lymphatics were located.

This suggests granulomatous lymphangitis as the underlying physiopathological mechanism of CD.

Our finding in third group showed (oral Roflumilast) aggregation of lymphocytes was located in the propria submucosa. Some colonocytes showed desquamation but were few compared to the control positive group.

Our finding in fourth group showed (rectal Roflumilast) congested blood vessels in the propria submucosa. Accumulation of lymphocytes was located in the propria submucosa and some large nodules were located in the mucosa-submucosa. Some crypts of liberkhum showed ulceration and accumulation of leucocytes.

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are aetiologically idiopathic, chronic, relapsing, and refractory inflammatory conditions that results from the interactions of gene susceptibility, environmental factors, disturbance of immune homeostasis, and microbiological anomaly in the gastrointestinal tract (Sartor, 2006).

TNBS instillation included focal necrosis of the mucosa, erosion, loss of goblet cells, and submucosal edema characterized by high level of inflammatory cell infiltration. The combination of A-PL and sulfasalazine afford protection against TNBS induced colonic damage (Yousefi et al., 2020).

Colonic inflammation involves the disruption of the apparatus of colonic mucosa and ulceration, resulting in the infiltration of inflammatory cells such as inflammatory monocytes and macrophages and thickening of the lamina propria (Buchheister et al.,2017).

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THE PARASITES OF SOME ASIAN CARP SPECIES FROM THE AQUATIC BIOTOPES FROM THE REPUBLIC OF MOLDOVA

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Abstract

As a result of the parasitological examination of some Asian carp species (common carp, prussian carp, silver carp, bighead carp) from a few fish ponds from Fălești district, 14 species of parasites were found, systematically classified into 7 classes, 12 families, 14 genera. The degree of infestation varied depending on the species: *Trichodina* sp. - common carp (EI-53.84%, II 1-10 ex.); *Dactylogyrus* sp. - common carp (EI-92.0%, II-1-45 ex.), prussian carp (EI-100%, II-1-45 ex.), silver carp (EI-72.7%, II-12-81 ex.), bighead carp (EI-100%, II-74-160 ex.); *Eudiplozoon nipponicum* - common carp (EI-38.46%, II-1-7 ex.), prussian carp (EI-16.0%, II-1-2 ex.); *Khawia sinensis* - common crap (EI-46.15%, II-1-15 ex.); *Schyzocotyle acheilognathi* – bighead carp (EI-9.09%, II-1 ex.); *Valipora campylancristrota* - common carp (EI-7.69%, II-1 ex.), silver carp (EI-9.09%, II-1 ex.), bighead carp (EI-44.4%, II-1-3 ex.); *Aspidogaster limacoides* – prussian carp (EI-4.0%, II-1 ex.); *Diplostomum spathaceum* - common carp (EI-30.76%, II-1-4 ex.), prussian carp (EI-20%, II-1-8 ex.), silver carp (EI-54.5%, II-1-15 ex.), bighead carp (EI-100%, II-1-92 ex.); *Posthodiplostomum cuticola* - common carp (EI-7.69%, II-1-2 ex.), silver carp (EI-54.5%, II-5-12 ex.); *Phillodistomum* sp. – prussian carp (EI-0.25%, II-1 ex.); *Phylometroides sanguinea* – prussian carp (EI-4.0%, II-1-2 ex.); *Sinergasilus lieni* – silver carp (EI-72.7%, II-7-26 ex.), bighead carp- (EI-100%, II-5-24 ex.); *Lernaea* sp. – common carp (1 ex.); Glochidium - common carp (EI-7.69%, II-1-10 ex.), prussian carp (EI-4.0%, II-4 ex).

Key words: (parasites, common carp, prussian carp, silver carp, bighead carp)

INTRODUCTION

In fish farming the mass death of both juveniles and adult fish is often recorded. This fact is closely related to the overpopulation of ponds with fish, which favors the accumulation of a large number of pathogens that can cause epizootics. Moreover, the infested fish can cause damage to other fish ponds as long as it is used to populate them (Gorchakova, 2000; Golovyna, 2014).

Among the diseases of fish, a great importance belongs to the invasive diseases, produced by parasitic agents with a great taxonomic diversity. The most common are parasites produced by protists (flagellates, ciliates, sporozoans, cnidosporidians) and those produced by worms or helminths (monogeneans, trematodes, cestodes, nematodes and acanthocephalans). These parasites parasitize both fish in natural and artificial waterbodies (Vasylkov, 1983, 1989).

Given the economic importance of fish, our goal was to perform the parasitological examination in order to detect potentially dangerous species for both fish and humans.

MATERIALS AND METHOD

The parasitological research was carried out in the laboratory of Parasitology and Helminthology of the Institute of Zoology, and performed according to the standard method proposed by Skryabin K.I. (1928) and the method proposed by Dogel and modified by Bykhovskaya Pavlovskaya (1985). The determination of the helminths was done acording to Bauer (1985, 1987). The microscopy of the detected helminths was performed using the stereomicroscope MBS, as well the examination at the optical microscope Novex Holland B, as fresh preparation slide-coverglass, with the objective 4x, 10x, 20x and ocular WF10X DIN/20MM. The detected nematodes were stored in Barbagallo solution (3% formaldehyde and 0,9% sodium chloride), and the trematodes were stored in 70% ethanol. For the parasitological evaluation, extensivity (%) and intensivity of invasion were used.

RESULTS AND DISCUSSIONS

As a result of the parasitological examination of 4 species of fish (common carp, prussian carp, silver carp, bighead carp) species of parasites with various locations, systematically classified in-

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to 7 classes, 12 families, 14 genera were detected (*table 1*).

Table 1

The parasites found in the examined Asian carp specimens

Class	Family	Species
Oligohymenophorea	Trichodinidae Claus, 1951	*Trichodina* sp.
Monogenea	Dactylogyridae Bychowsky, 1933	*Dactylogyrus* sp.
	Diplozoidae Palombi,1949	*Eudiplozoon nipponicum* (Goto, 1891)
Trematoda	Diplostomidae Poirier, 1886	*Diplostomum spathaceum* (Rudolphi, 1819) Olsson, 1876
		Posthodiplostomum cuticola (von Nordmann, 1832) Dubois, 1936
	Gorgoderidae Looss, 1899	*Phillodistomum sp.*
	Aspidogastridae Poche, 1907	*Aspidogaster limacoides* Diesing, 1834
Cestoda	Lytocestidae Hunter, 1927	*Khawia sinensis* Hsü, 1935
	Bothriocephalidae Blanchard, 1849	*Schyzocotyle acheilognathi (Yamaguti, 1934)* Brabec, Waeschenbach, Scholz, Littlewood & Kuchta, 2015
	Gryporhynchidae Blanchard, 1849	*Valipora campylancristrota* (Wedl, 1855)
Enoplea	Philometridae Baylis & Daubney, 1926	*Philometroides sanguinea* (Rudolphi, 1819)
Hexanauplia	Lernaeidae Cobbold, 1879	*Lernaea* sp.
	Ergasilidae Burmeister, 1835	*Sinergasilus lieni* Yin, 1949
Bivalvia	-	Glochidium

As a result of the examination of the gill lamellae, helminths from the class Monogenea, genera *Dactylogyrus* (*figure 1*) and *Eudiplozoon* were detected. From the genus *Dactylogyrus*, the most dangerous to fish are *Dactylogyrus vastator*, *D. extensus* and *D. anchoratus*.

Figure 1. **Specimen of *Dactylogyrus* sp. detected on the gills of prussian carp**

The representatives of this genus are worms with a flattened body, 0.75-1 mm long, 0.15-0.38 mm wide, that ataches to the epithelium of the gill lamellae of various fishes, through a complex adhezive organ called opisthaptor, which is armed with 2 large central hooks and 14 small hooks arranged marginally (*figure 2*). The shape, size of the hooks, and the attachment disc, vary depending on the species, and are an important taxonomic feature for determination (Gibson, 1986).

The performed parasitological examination revealed the infestation with *Dactylogyrus* sp. of the following fish species: common carp (EI-92%, II-1-45ex.), silver carp (EI-100%, II-1-45 ex.), silver carp (EI-72.7%, II- 12-81 ex.), bighead carp (EI-100%, II-74-160ex.).

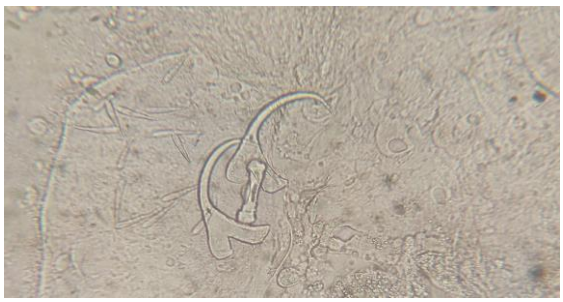

Figure 2. **Large hooks and marginal hooks of *Dactylogyrus* sp.**

One of the representatives of the genus *Eudiplozoon* is *E. nipponicum* (*figure 3*). This species parasitizes on gill lamellae of common carp, crucian carp, bream and other cyprinids. It is a hematophagous parasite which, according to Kawatsu (quoted by Bauyer, 1985), in case of massive infestations, can cause hypochromic anemia.

The performed parasitological examination revealed the infestation with *E. nipponicum* of the following fish species: common carp (EI-38.46%, II-1-7ex.), prussian carp (EI-16.0%, II-1- 2 ex.).

Figure 3. ***Eudiplozoon nipponicum*** **detected on the gill lamellae of comon carp**

Also, as a result of the parasitological examination of the gill lamellae of common carp, protists from the genus *Trichodina* were detected (*figure 4*). The extensivity of invasion reached 53.84% and the intensivity of the invasion 1-10 specimens in a microscope field of view.

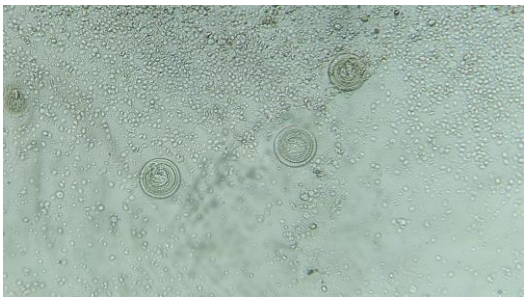

Figure 4 ***Trichodina*** **sp. detected on the gills of common carp**

Parasitological examination of the gills of common carp and prussian carp revealed the presence of parasitic larval stages of freshwater mussels, aquatic bivalve mollusks, from the genera *Unio*, *Anodonta*, *Margaritana*, known also as glochidium (*figure 5*). During the parasitological examination, glochidia were detected in common carp and prussian carp. In the case of the common carp, the extensivity of invasion with glochidia was 7.69%, and the intensivity of invasion 1-10 specimens. In the case of silver carp the extensivity of invasion was 4.0%, and the intensivity of invasion 4 specimens.

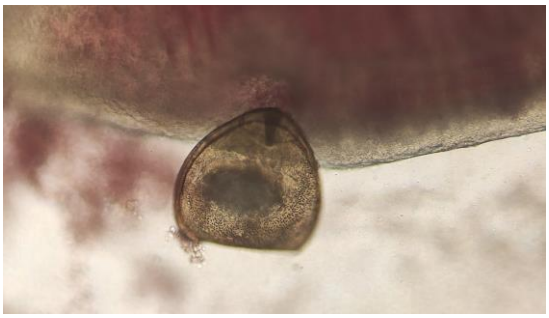

Figure 5. **Glochidium on the gills of a common carp specimen**

From the group of parasitic crustaceans *Sinergasilus lieni* and *Lernaea* sp. were detected (only one specimen of *Lernaea* sp. was detected on a common carp, attached to the ventral aorta).

S. lieni (*figure 6*) is a parasitic crustacean, specific to silver carp and bighead carp, that parasitizes on the gills. In case of a high degree of infestation, *S. lieni* cause inflammation, necrosis of the gills, poor blood circulation and gas metabolism that results in asphyxiation of the infested fish.

In the carried parasitological research, the extensivity of invasion of silver carp with *S. lieni* was 72.7%, and the intensivity of invasion – 7-26 specimens. In bighead carp the parasite was found in 100% of cases, and the intensivity of invasion was 5-24 specimens.

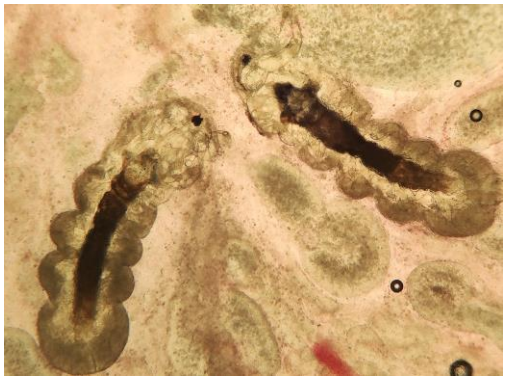

Figure 6**. Specimens of *Sinergasilus lieni* detected on the gills of bighead carp**

From de Diplostomidae family, trematode species *Diplostomum spathaceum* and *Posthodiplostomum cuticola* were found. *D. spathaceum* (*figure 7*) is a trematode, the larval stage (metacercariae) of which causes a very dangerous disease called diplostomiasis or ,,white eye" disease. Diplostomiasis, depending on age, can develop acute and chronic. Specific for the chronic form are the lesions of the eye lens, manifested by cataract. In chronic form, due to cataract, the fish does not feed enough and, respectively, the body weight decreases, compared to that of healthy fish (Shigin, 1976).

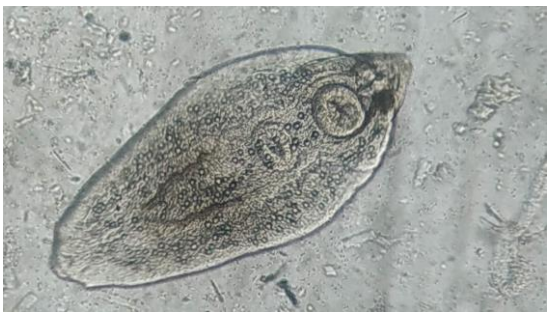

Figure 7.***Diplostomum spathaceum*** **– metacercariae found in the lens of common carp**

The infested fish swim more on the surface of the water, thus being predisposed to be eaten by

piscivorous birds. In the acute form, the infested fish is agitated, does not feed and has punctiform hemorrhages on the opercula and in the eyeballs.

In the examined fishes, *D. spathaceum* was found in common carp (EI-30.76%, II-1-4 ex.), prussian carp (EI-20.0%, II-1-8 ex.), silver carp (EI-54.5%, II-1-15ex.), and bighead carp (EI-100%, II-1-92ex.).

The second species of trematodes from family Diplostomidae found in the examined fishes is *Posthodiplostomum cuticola*, which causes an invasive disease called postodiplostomiasis (*figure 8*), which is characterized by the initial appearance of black spots of various size on the fish skin, which then turns into small cysts surrounded by melanic pigment - hemomelanin, which is the product of the degradation of hemoglobin and chromatophores. Inside the cyst the metacercariae is located (*figure 9*). Spots and cysts appear in different regions of the body: on the fins, gills, caudal peduncle, in the dorsal and lateral region of the body, on the cornea, in the oral cavity, and their number can vary from a few tens to several hundred. In case of a massive infestation, the deformity and the loss of the flexibility of the spine can be noticed. The fish remains undeveloped, being predisposed to be caught by the piscivorous birds (Goloshchapova, 2014).

Infested with *P. cuticola* were common carp and silver carp. The extensivity and intensivity of invasion in common carp were 7.69%, 1-2 ex., and 54.5%, 5 -12ex. in silver carp.

Figure 8. **Silver carp infested with *Posthodiplostomum cuticola***

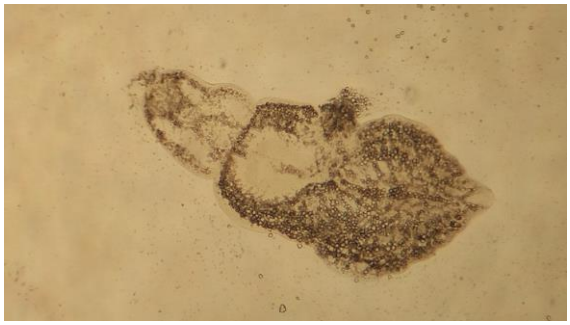

Figure 9. ***Posthodiplostomum cuticola* – metacercariae extracted from the cyst**

Also, as a result of the inspection of the prussian carp tegument, the nematode *Philometroides sanguinea* (*figure 10*) was detected. The location of the parasite in the host differs. Males parasitize inside the abdominal cavity, between the walls of swim bladder, and females between the bony rays of the caudal fin, less often the dorsal fin.

The extensivity of invasion with *P. sanguinea* was 4.0%, and the intensivity of the invasion 1-2 specimens.

Figure 10. **Prussian carp infested with *Philometroides sanguinea***

During parasitological examination 4 species of parasites were detected in the organs of the digestive system: *Khawia sinensis*, *Schyzocotyle acheilognathi*, *Aspidogaster limacoides* in the intestine, and *Valipora campylancristrota* in the gallbladder.

K. sinensis (*figure 11*) is a flatworm from the class Cestoda, with a white, non-segmented body, 80-175 mm long and 2.5-3.5 mm wide, specific to common carp and grass carp. The parasite can be found in all fish ponds were common carp is farmed, susceptible to infestation being all age groups. However, the extensivity and intensivity of invasion in adult fishes is lower than in youth. Also, the degree of infestation with this helminth is influenced by the environmental conditions. In water bodies with a stagnant, muddy water, preferred by aquatic oligochaetes (intermediate hosts), the level of fish infestation is much higher (Mirzoyeva, 2016).

Figure 11. **Specimens of *Khawia sinensins* in the intestine of common carp**

As a result of the parasitological examination, *K. sinensis* was detected in common carp. The extensivity of the invasion with *K. sinensis* was 46.15% and the intensivity of the invasion 1-13 specimens.

S. acheilognathi (*figure 12*) is a cestode that can infest various cyprinids, such as common carp, prussian carp, bighead carp, etc. The most susceptible to the infestation are common carp and bighead carp youth. In these species the extensivity of invasion can reach 80-100%. This parasite is allogen for the aquatic biotopes in the Republic of Moldova. He entered the water bodies via grass carp, brought for acclimatization in the early 60s of the 20th century (Marits, 1968). The causes of *S. acheilognathi* invasion can be: the adult infested fish, which act as vector, the simultaneous farming of fish of different age groups, the invasion of fish ponds with infested crustaceans (Bauyer, 1981).

As a result of the parasitological examination *S. acheilognathi* was detected in bighead carp. The extensivity of invasion was 9.09%, and the intensivity invasion 1-2 specimens.

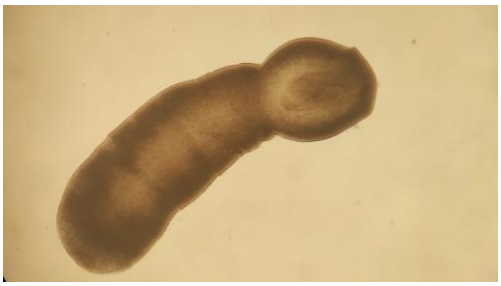

Figure 12. ***Schyzocotyle acheilognathi*** **detected in the intestine of bighead carp**

From the species of trematodes, that parasitize the digestive system, *A. limacoides* was detected (*figure 13*). Out of the total examined fishes, only prussian carp was infested. The extensivity of invasion of prussian carp with *A. limacoides* was 4.0%, and the intensivity of invasion 1-2 specimens.

Figure 13. ***Aspidogaster limacoides*** **detected in the intestine of prussian carp**

After the examination of gallbladder of the captured fishes, cestodes from the family Gryporhynchidae, represented by the larval stage (plerocerc) of *Valipora campylancristrota* (*figure 14*) were found.

Infested with *V. campylancristrota* were common carp (EI-7.69%, II-1 ex.), silver carp (EI-9.09%, II-1 ex.), and bighead carp (EI-44.4%, II-1-3 ex.).

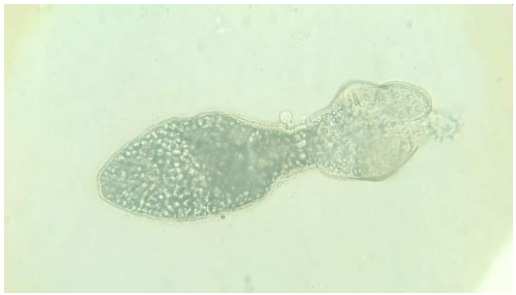

Figure 14. ***Valipora campylancristrota*** **detected in the gallbladder of bighead carp**

During the parasitological examination of the organs of the urinary tract (ureters, urinary bladder) of prussian carp, trematodes from the family Gorgoderidae, genus *Phillodistomum* (*figure 15*) were detected.

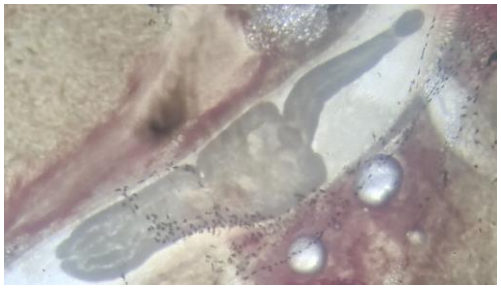

Figure. 15 ***Phillodistomum*** **sp – detected in the ureter of prussian carp**

The extensivity of invasion of prussian carp with *Phillodistomum* sp. was 0.25%, and the intensivity of invasion 1 specimen.

CONCLUSIONS

As a result of the parasitological examination of the studied fish species, 14 species of parasites were detected. Out of the total detected species, 2 were specific: *Sinergasilus lieni* specific for silver carp and bighead carp, and *Philometroides sanguinea* specific for prussian carp.

The highest extensivity of invasion was recorded in the case of infestation with monogenetic trematode *Dactylogyrus* sp. in the following species: common carp (EI-92%), prussian carp (EI-100%), silver carp (EI-72.7%), bighead carp (EI-100%), and the highest intensivity of invasion with this parasite was recorded in bighead carp (II-160ex.).

In the case of trematodes, the highest prevalence showed *Diplostomum spathaceum* found in the following species: common carp (EI-30.76%),

prussian carp (EI-20.0%), silver carp (EI-54.5%), bighead carp (EI-100%). The highest intensivity of invasion with *D. spathaceum* was recorded in bighead carp (II-92 ex.).

In the case o cestodes, the highest prevalence showed *Khawia sinensis* found in common carp (EI-46.15%).

ACKNOWLEGMENTS

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EVALUATION OF EPIDURAL ANESTHESIA WITH LIDOCAIN COMPARED TO BUPRENORPHINE AND THE COMBINATION OF LIDOCAIN - BUPRENORPHINE IN DOGS

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Abstract

The aim of our study is the comparative evaluation of epidural anesthesia by determining the antinociceptive efficacy and the modification of cardiorespiratory variables, in dogs in which a local anesthetic (lidocaine) and an opioid analgesic (buprenorphine) were injected into the epidural space separately and in combination. The solutions were administered in the epidural space to the dog as follows: lidocaine 2% (2.5 mg / kg) and buprenorphine (concentration to be added) (1.5 mcg/kg), respectively lidocaine 2% (5 mg/kc) or buprenorphine 0.3 mg/ml (3 mcg/kc). Lidocaine had excellent penetrability, rapidly producing onset (1.7 ± 0.30 min.) and onset of surgical anesthesia. However, it did not induce long-lasting sensory and motor nerve block and no satisfactory analgesia (6.0 ± 0.1.). Buprenorphine had a slow onset, but with a long-lasting analgesic effect (108.0 ± 41.6 min.). Epidural administration of opioids provides additional intra- and postoperative analgesia, in which case buprenorphine may be the drug of choice in laborious surgical procedures**.**

Key words: epidural anesthesia, lidocaine, buprenorphine, analgesia

INTRODUCTION

Preoperative epidural injection of local anesthetics and opioids provides excellent preemptive, multimodal intraoperative analgesia, reduces the concentration of volatile anesthetic required to maintain surgical anesthesia, and provides analgesia extending into the recovery period. Epidural administration of drugs for pain management has been widely used in veterinary medicine (*Jones RS., 2001; Steagall PVM et al., 2017*).

The lumbosacral intervertebral space is the most common location for epidural injection in small animals. Epidural administration of local anesthetics provides complete anesthesia, sufficient to perform surgery, to the caudal half of the body. Administering opioids epidurally provides additional analgesia often of longer duration and with fewer adverse effects than systemic administration. Lumbosacral epidural anesthesia with local anesthetics provides complete anesthesia to the caudal half of the body by blocking the intradural spinal nerve roots and the peripheral layer of the spinal cord (*Stoelting RK, Hillier SC, 2006*). This technique is useful for pelvic and hindlimb orthopedic procedures, perineal and anal surgeries, exploratory laparotomy, and cesarean section. In addition to complete anesthesia, at least some sympathetic and motor blockade is produced with local anesthetics. Lidocaine, mepivacaine, and bupivacaine consistently cause motor blockade, while motor blockade is less intense and of shorter duration with levobupivacaine and ropivacaine (*Stoelting RK, Hillier SC, 2006*). The duration of motor blockade is generally shorter than the duration of analgesia, and, depending on the procedure and local anesthetic chosen, motor function usually returns by the time a patient recovers from anesthesia (*Lemke KA., 2007*; *Scarda RT, Tranquilli WJ*., 2007; Stoelting RK, Hillier SC, 2006).

MATERIAL AND METHOD

The study was performed on 15 healthy common breed dogs (13 males and 2 neutered females). They weighed an average of 10.8 ± 1.4 kg and were kept in identical conditions during the experiment. The dogs received water ad libidum (at discretion), and 12 hours before the experiment they were stopped feeding. Also, before starting the experiment, they underwent a rigorous physical and biochemical examination. The commercial products used were: Lidocaine®, 2% solution with 1: 200,000 epinefrine in 20 ml vials and Bupaq, 0.3 mg / ml (0.003%) solution.

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A catheter was inserted into the antebrachial cephalic vein to collect blood samples to determine blood gas, arterial and venous pH (pHa, pHv), concentration in arterial and venous hemoglobin (Hg, g/dl), and others parameters using the AVL Compact 2 gas analyzer. A non-invasive indirect automatic device (Digital Electronic Blood Pressure Monitor, Model FC - 150 D, Tokyo, Japan) was used to measure blood pressure and heart rate. Respiratory rate was assessed by observing and counting chest "trips". Rectal temperature was measured in degrees Celsius (0C), using an electronic thermometer (Termir® 4 Electronic Veterinary Thermometer).

The dogs did not receive water and food 6 hours before the anesthesia. They were placed in lateral decubitus, after being pre-anesthetized with acepromazine, 1% solution - 0.5 mg / kc, i.m., in ventroflexion to highlight the lumbar vertebral space. The craniodorsal iliac spine, the dorsal spinal process of the L7 vertebra and the median sacral ridge were palpated. Asepsis followed the rules of any surgical procedure (the lumbosacral region was trimmed, shaved and aseptically prepared for each dog).

Experimental Design: the puncture needle (i.v. catheter 18GA 1.77IN - 1.3 x 45 mm) is inserted in the center of the lumbosacral space perpendicular to the midline of the kidney and in a strictly sagittal plane, using the left index as a guide. The catheter is inserted 1 - 1.5 cm, and after the needle is withdrawn, a syringe with 7 ml of sterile isotonic saline (0.9% NaCl) is attached to the cannula to tactilely assess the resistance during the progression of the needle. enlarges suddenly when the needle penetrates the yellow ligament. After an advance of a few millimeters, a sudden loss of resistance indicates penetration into the epidural space. The injection of the solutions into the epidural space was done slowly over a period of at least 30 seconds.

Each dog was randomly given 3 treatments: lidocaine (5 mg/kg), bupivacaine (3 mcg/kg) and a combination of the two in a half dose: lidocaine (2.5 mg/kg) and bupivacaine (1.5 mcg/kg). A time interval of at least 14 days was observed between administrations, each anesthetic being appropriately diluted with an injection volume of 3 ml with isotonic saline. The installation of sensory blockage (analgesia) was assessed by the response to the pressure applied on the digital perineum, perineum and flanks with a hemostatic forceps (closed at the first teeth). The initiation and installation of motor blockage was appreciated by the feeling of general weakness or by the inability of the dog to carry the weight of its hind limbs, the absence of skeletal muscle tone.

Lateral decubitus time, onset of analgesia, duration of analgesia, and recovery time from anesthesia (sternal decubitus period) were measured for each dog. Heart rate, mean blood pressure, respiratory rate, rectal temperature, and blood biochemical tests were measured before the epidural injection and then at 15-minute intervals for a 75-minute post-injection period.

Data are presented as M ± DS. Anesthetic indices were compared using the Student's test. The ANOVA test (analysis of variance) was used to compare measurements of physiological indices: heart rate, respiratory rate, mean blood pressure, rectal temperature and blood biochemical indices. For all analyzes a value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Results

After mixing the solutions of lidocaine and buprenorphine in the same syringe, no changes were observed to indicate an incompatibility (precipitation reaction). Dogs were pre-anesthetized with acepromazine 0.5 mg / kc. Epidural puncture was more difficult to perform in 2 of the 15 dogs, which were more resistant to the pre-anesthetic administered (20 minutes before restraint) and resisted manual restraint.

The anesthetic indices of epidural anesthesia performed with lidocaine, buprenorphine and the lidocaine - buprenorphine mixture are presented in table 1.

The decubitus time with buprenorphine and the lidocaine-buprenorphine mixture was longer than with lidocaine. Although there were no significant differences in the onset of analgesia with the three anesthetic protocols, the longest duration of analgesia was recorded with the lidocaine-buprenorphine mixture, the shortest in lidocaine and the medium in buprenorphine.

The averages of heart rate, mean blood pressure, respiratory rate and rectal temperature for each of the three treatment groups are shown in Tables 2, 3, 4 and 5.

There were no significant changes in the measured physiological variables.

Discussions

The administration of anesthetic agents in the epidural space is influenced by numerous factors that must be controlled, especially in this

study (age, obesity, etc.) (*Garcia-Pereira F., 2018; Smith L.J., Yu J.K., 2001*).

In clinical practice, dogs should normally be sedated (which we did using a neuroleptic phenothiazine - acepromazine 0.5 mg / kc) to facilitate epidural puncture *(Caniglia A.M. et al., 2012; Bauer M.C., 2014)*. The use of stronger preanesthetic (e.g. thiopental, xylazine, etc.) was deliberately omitted in this study to avoid the influence of these agents on physiological variables.

Epidural blockage could not be performed in two dogs due to the dislocation of the needle from the epidural space due to the agitation of the dog, which did not calm down properly. In this study it was shown that the solutions of lidocaine and buprenorphine in the mixture are miscible, thus indicating the existence of a pharmaceutical compatibility.

Epidural administration of lidocaine had the fastest lateral decubitus installation time of 1.7 ± 0.3. This is related to the excellent diffusion property and penetrability of this anesthetic agent under study (*Campoy L. 2004; Hall L.W., Clarke, K.W., 1991; Stein C. et al., 2001*).

The type and volume of local anesthetic chosen depends on the desired result. Lidocaine (2%) administered at 1 ml/6 kg completely anesthetizes the pelvic limbs and posterior abdomen, caudal to L1, within 10 to 15 minutes and lasts 60 to 120 minutes. With a comparable volume of 0.5% to 0.75% bupivacaine, the onset is 20 to 30 minutes with a duration of four to six hours. Volumes of 1 ml/7.5 kg are adequate for pelvic, perineal, and hind limb procedures (Hansen B.D., 2001).

The onset of analgesia, under the three anesthetic techniques, was not significantly different, the total duration of analgesia was longer in the case of the lidocaine-buprenorphine mixture (137.6 ± 32.9). This suggests that lidocaine and buprenorphine acted synergistically.

All parenteral formulations of buprenorphine are preservative-free and safe for epidural use (*Valverde A., 2008*). A prolonged duration of analgesia allows the performance of major diagnostic procedures, surgical and obstetric procedures, using a single anesthetic dose. The recovery time (sternal decubitus period or immediate recovery) was recorded as the longest (184.0 ± 1.5) in the case of buprenorphine epidural anesthesia, making this anesthetic unsuitable for use in situations where wants a speedy recovery.

It should be noted that the epidural administration of lidocaine, buprenorphine, lidocaine-buprenorphine mixture in dogs does not produce significant changes in heart rate, mean blood pressure, respiratory rate and body temperature. This is in line with previous reports (*Hansen B.D., 2001*) on physiological indices in buprenorphine epidural blockages in dogs.

This apparent physiological stability tells us the existence of a compensatory homeostatic mechanism in non-pre-anesthetized or slightly pre-anesthetized dogs (in our case), healthy or under the influence of other factors.

CONCLUSIONS

1. Buprenorphine may offer certain benefits for epidural use, such as a lower potential for abuse and, in some clinics, lower costs and less drug waste.

2. Epidural administration of the combination lidocaine - buprenorphine in dogs causes a strong analgesia, important for animals likely to show central hypersensitivity, a source of hyperalgesic responses and when postoperative analgesic treatment cannot neutralize the pain alone.

3. It reduces by almost 50% the amount of anesthetic agent, being characterized, in fact, by a potentiating synergistic effect.

4. This combination also causes a long-lasting analgesia of about 2 hours and excellent muscle relaxation.

5. In addition to the intra- and postoperative antinociceptive efficacy, it is observed that there were no significant respiratory and cardiac side effects and no changes in body temperature.

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Table 1.

Mean values (± SD) of the anesthetic indices (min.) Of lidocaine, buprenorphine and the combination lidocaine - buprenorphine in dogs

Anesthetic indexes	Lidocaine (a)	Buprenorphine (b)	Combination lidocaine - buprenorphine (c)
Decubitus time	1,7 ± 0,3	2,8 ± 0,7^{+}	4,8 ± 1,9^{+}
Onset of analgesia	6,0 ± 0,0	6,0 ± 1,0	6,2 ± 1,5
Duration of analgesia	83,0 ± 9,3	108,0 ± 41,6^{+}	137,6 ± 32,9^{+}
Recovery time	48,9 ± 4,0	184,0 ± 1,5	51,8 ± 12,9

Data are presented as M ± DS, + = significant differences ($p < 0.05$)

Table 2

Mean heart rate (± SD) (HR; beats / minute)

Time interval (minutes)	Lidocaine (a)	Buprenorphine (b)	Combination lidocaine - buprenorphine (c)
0	101,4 ± 5,4	116,3 ± 14,8	139,8 ± 9,8
15	86,2 ± 6,0	97,0 ± 9,9	125,0 ± 19,2
30	83,9 ± 13,6	104,0 ± 12,4	115,4 ± 12,9
45	83,7 ± 16,1	103,4 ± 14,1	117,4 ± 13,0
60	84,2 ± 13,1	102,6 ± 9,9	111,8 ± 13,6
75	90,1 ± 15,8	103,0 ± 11,4	108,0 ± 9,0

Data are presented as M ± SD

Table 3.

Mean values (± SD) of mean blood pressure (MBP; mmHg)

Time interval (minutes)	Lidocaine (a)	Buprenorphine (b)	Combination lidocaine - buprenorphine (c)
0	134,8 ± 22,3	112,0 ± 5,2	114,0 ± 10,7
15	81,3 ± 14,2	101,6 ± 7,6	111,0 ± 10,7
30	103,4 ± 13,6	104,8 ± 8,5	102,1 ± 8,1
45	112,3 ± 11,4	105,3 ± 9,1	103,1 ± 8,6
60	99,2 ± 10,3	106,6 ± 9,1	112,5 ± 16,3
75	91,4 ± 12,4	94,5 ± 6,5	99,5 ± 12,2

Data are presented as M ± SD

Table 4.

Mean values (± SD) of respiratory rate (RR; breaths / minute)

Time interval (minutes)	Lidocaine (a)	Buprenorphine (b)	Combination lidocaine - buprenorphine (c)
0	30,6 ± 4,8	22,6 ± 3,0	26,2 ± 6,8
15	19,4 ± 4,3	20,6 ± 2,4	19,4 ± 3,6
30	17,8 ± 4,3	19,8 ± 5,0	19,6 ± 2,9
45	19,1 ± 6,4	17,7 ± 2,0	19,5 ± 3,7
60	22,8 ± 9,1	17,4 ± 2,7	17,9 ± 4,0
75	24,6 ± 13,8	16,1 ± 2,9	18,2 ± 4,2

Data are presented as M ± DS

Table 5.

Mean body temperature values (± SD) (0C)

Time interval (minutes)	Lidocaine (a)	Buprenorphine (b)	Combination lidocaine - buprenorphine (c)
0	38,6 ± 0,2	39,0 ± 0,4	39,6 ± 0,3
15	39,0 ± 0,2	38,7 ± 0,3	39,4 ± 0,2
30	39,1 ± 0,2	38,8 ± 0,2	39,5 ± 0,3
45	38,7 ± 0,5	38,7 ± 0,1	39,6 ± 0,3
60	38,4 ± 0,6	38,5 ± 0,1	39,2 ± 0,3
75	38,2 ± 0,5	38,3 ± 0,1	39,0 ± 0,3

Data are presented as M ± SD

EFFECT OF LONG-TERM EXPOSURE TO NON-THERMAL PLASMA ACTIVATED WATER ON METHEMOGLOBIN IN MICE

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Abstract

Non-thermal plasma activated water (PAW) is described as a potent antimicrobial agent, but although it has numerous bio-medical applications, there is a lack of toxicity studies in living organisms. Thus, as a main objective, we aimed to evaluate the *in vivo* methemoglobin sizing potential of non-thermal plasma activated water (PAW) in CD-1 mice. The device used in our experiment is based on the GlidArc principle, with the advantage of adjusting the values of the current in the circuit on account of a special power supply, which works with magnetic scattering fluxes. A daily volume of 300 ml of PAW was prepared daily with this reactor with the following physico-chemical parameters: conductivity 446 ± 25 μS / cm, pH 2.78 ± 0.12, ORP + 1.06 V, NO2-192 ± 10 mg / L, NO3-1550 ± 95 mg / L, H2O2 2.6 ± 0.12 mg / L, O3 1.08 ± 0.07 mg / L, peroxynitrite - ONOO-. After analysis and interpretation of the data, it was found that methemoglobinemia did not differ significantly in the groups treated with PAW ($p < 0.05$) compared to the control group ($p = 0.8076$). Thus, long-term consumption of PAW has no detrimental effects on the health status of CD-1 mice.

Key words: PAW, mice, methemoglobin

INTRODUCTION

In physics, the term "plasma" refers to the fourth state of aggregation of matter that exists in nature in a wide variety of forms and can be artificially created in different ways, known in technology as thermal plasma and non - thermal type (the gas used remains at a low temperature).

Due to the diversity of fields of application (industrial, bio-medical) the generation of a certain type of plasma and the specific requirements of each application (temperature, potential, chemical composition, flow, etc.) depend on the selection of the source.

Most plasma sources cannot operate in a wide range of parameters, so the operating conditions must be adapted to each application. Technologically, the most commonly used method of generating plasma at low temperatures is to apply an electric field to a neutral gas (Conrads H., Schimidt M., 2000).

An increasing number of original articles discuss numerous applications of non-thermal plasma in biology and medicine. For example, this type of plasma has been used with very good results in decontamination and sterilization of medical devices (Weinstein R.A., 2011), in the treatment of periodontal disease (Sun S.Y. et al, 2016), dermatology (Bogle M.A. et al., 2007), microbiology and many other fields (Laheij A.M.G.A. et al., 2012; Fridman G. et al., 2007).

In dentistry, treatment with cold plasma as a source of reactive species was superior to that with chlorhexidine in the removal of periodontal biofilm (Nayansi Jha, et al., 2017; Sun S.Y. et al., 2016).

Particular attention was paid to the use of non-thermal plasma in contact with liquids (PAL-Plasma activated liquid, including PAW-Plasma activated Water) (Brisset J.L., et al., 2008).

Water treatment with non-thermal plasma induces structural changes (water becomes mono-molecular by breaking hydrogen bonds) and generates numerous reactive species of oxygen and nitrogen, the most important being hydrogen peroxide and peroxynitrite, both showing a strong antimicrobial effect, with technological implications in the medical field, especially decontamination and sanitation.

In our study we aimed to evaluate the *in vivo* methemoglobin sizing potential of non-thermal plasma activated water (PAW) in CD-1 mice, watered 4 hours daily for 90 days.

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MATERIAL AND METHOD

The device used in our experiment is based on the GlidArc principle. PAW preparation requires the blowing of atmospheric air at high speed (10 m/s), supplied by an air compressor and injected through a stainless-steel nozzle into the plasma area produced by the electric discharge in order to maintain the character of cold plasma and to create a turbulent regime on the water surface, to have an efficient mixing of reactive species in water.

The operating conditions of this cold plasma generator have been previously described by Hnatiuc et al., 2014. PAW was prepared in a cylindrical, electro-insulated glass device, in which two metal electrodes with divergent profiles are placed, between which the useful electric discharge can be manifested.

Cold plasma activated water was prepared in the parameters: 446 ± 25 μS / cm, pH 2.78 ± 0.12, ORP +1.06 V, NO2-192 ± 10 mg / L, NO3-1550 ± 95 mg / L, H2O2 2.6 ± 0.12 mg / L, O3 1.08 ± 0.07 mg / L. The average value of the power used to prepare the water was calculated at 111.6 W.

The scheme of the plasma generator is shown in Figure 1.

Fig. 1. Configuration of the non-thermal plasma generator based on a gliding arc discharge

In our study were used 100 CD-1 mice, purchased 2 weeks before the start of the experiment at the Cantacuzino Institute Bucharest, Baneasa resort, 10-week-old nulliparous females, with a mean weight at the beginning of the experiment of 28.85 ± 5.12 grams. The acclimatization of the mice was done under identical conditions of temperature (22 ± 0.7°C) and humidity (50 ± 10%), and the light/dark cycle provided was 12 hours.

Each experimental group was housed in autoclavable polycarbonate cages of 1500 cm2, approximately 300 cm^2/mouse. The animals had a permanent access to water (ad libitum) (autoclavable bottles with drip system) and standardized food at Cantacuzino Institute, with the following composition: 23% protein, 10% fat, 50% carbohydrates, 8% crude fiber and 9 % vitamin-mineral premix, calcium carbonate and phosphate, amino acids.

Mice were divided into two groups, namely the control group (M) watered with drinking water (n = 5) and the experimental group (E), watered with PAW (n = 5). The experiment took place over 90 days, during which time drinking water was replaced daily, at the same time, with PAW prepared under the same conditions. Thus, a volume of 300 ml of distilled water was treated every morning at 8 o'clock for 10 minutes, after which the treated water was passed into borosilicate bottles used for watering all animals. In order to guarantee that the mice consumed this water, they were deprived of running water 6 hours before the administration of PAW. Their watering with PAW was done only for 4 hours, daily, throughout the experiment.

The physico-chemical parameters of the distilled water used in our experiment in the preparation of PAW are the following: conductivity 5 ± 0.3 μS / cm, pH 6.5 ± 0.16, NO2 - undetectable, NO3- undetectable. The physico-chemical parameters for PAW are: conductivity 446 ± 25 μS / cm, pH 2.78 ± 0.12, ORP + 1.06 V, NO2-192 ± 10 mg / L, NO3-1550 ± 95 mg / L, H2O2 2.6 ± 0.12 mg / L, O3 1.08 ± 0.07 mg / L, peroxynitrite - ONOO-.

The two experimental groups were monitored daily, in the same time interval, for 90 days, clinically following: signs of general toxicity (hepatic, cardiac, pulmonary or renal adverse effects) and behavioral changes (reduced food intake, depression, and abnormal body posture).

The determination of the interaction between nitrates and hemoglobin (methemoglobinemia) was evaluated by spectrophotometry. To determine the degree of methemoglobinemia, each mouse was harvested by puncturing the submandibular vein 0.2 ml of blood. Then, 100 μl of blood was mixed with 100 μl of 1% saponin and homogenized to hemolyze the red blood cells, after which 6 ml of phosphate buffer M / 60 pH-6.8 was added.

The relative absorbance measurement was performed with a Boeco UV-vis UV

spectrophotometer at 630 nm. The percentage of methemoglobin is calculated according to the formula% MtHb = absorbance x 100. If the MtHb level was below 10%, it was considered safe for mouse and human health.

Statistical analysis

Statistical comparisons between two groups were evaluated using unpaired T TEST. All numerical data were presented as Mean ± SD. Significance was accepted at P> 0.05.

Ethical implications

The study was conducted in accordance with national and international legal regulations on animal welfare, identification, control and elimination of factors causing physiological and behavioral disorders: Directive EC86 / 609 EU.

RESULTS AND DISCUSSION

The data recorded in the two groups were 3,631 ± 0.46 in the control group (M) watered with drinking water and 3,695 ± 0.33 in the experimental group watered with PAW (p = 0.8076) (Table 1).

Table 1

Methemoglobin variations (%)

GROUP	MtHg (%) (M±SD)
Martor	3.631±0.46
PAW	3.695±0.33
p > 0.05	p=0.8076

Methemoglobin levels recorded in the experimental group (PAW) were not significantly different from those recorded in the control group (p> 0.05). The averages of these measurements are below the clinically tolerated human minimum of 10%, a level from which adverse health effects can be observed. No adverse effects were observed in mice during the 90 days of the experiment.

During the 90 days of adaptation of mice with PAW, no mortality or morbidity, adverse effects or other clinical and behavioral changes occurred, especially in terms of food and water consumption, they remained at the same level throughout the experiment.

The effect of PAW is determined by the type and concentration of reactive oxygen species (ROS) and nitrogen (RONS), which are generated depending on the chemical environment (gases and liquids used), the stress exerted and the method of preparation. The use of atmospheric air and distilled water in the production of PAW, leads to the formation of primary species (atomic oxygen, oxygen, superoxide, ozone, hydroxyl radicals and nitrogen atoms), which will then continue to interact and form secondary species (hydrogen peroxide , peroxynitrite, nitric oxide, nitrates and nitrite ions), species that will be distributed in the liquid mass, thus enriching the water in reactive species with antimicrobial role, especially peroxynitrite (Brisset J.L., Hnatiuc E., 2012; Brisset J. L., & Pawlat J., 2016).

Methemoglobin is formed by the oxidation of iron from hemoglobin. The oxidation reaction affects the ability of hemoglobin to carry oxygen, leading to tissue hypoxia and possibly death (Umbreit J., 2007). A small percentage of methemoglobin (1-2%) is commonly found in human and animal blood, but does not produce clinical signs. A level higher than 10% causes mucosal cyanosis, dyspnea, anxiety, fatigue, confusion, dizziness, tachypnea, convulsions and even death when the proportion of total hemoglobin is > 50. The causes are various, but among the most involved substances in the development of this condition we note nitrites and nitrates.

Due to the generous nitrite content of PAW, we expect the percentage of methemoglobinemia to vary significantly in mice given this water daily for 90 days. However, the methemoglobin levels recorded in the experimental group did not differ significantly from those recorded in the control group (p> 0.05). Moreover, the averages of these measurements are below the clinically tolerated human minimum of 10%, a level from which adverse health effects can be observed. No adverse effects were observed in mice during the 90 days of the experiment. The presence of an approximately similar percentage in the control group of methemoglobinemia can be explained by the interaction of chlorides in drinking water with hemoglobin. Balalau et al., 2005, stated that chlorations in mouthwashes and toothpastes induce a certain level of methemoglobinemia.

The absorption of the ingested nitrate ion is usually achieved in the upper segment of the small intestine, with a bioavailability of 100%, being quickly distributed in the body. A probable explanation for this low percentage of methemoglobin in the mice of the experimental group, adapted with PAW (<4%) is related to the interactions between reactive nitrogen species

(SRON) with the organic material in the digestive tract, thus neutralizing at this level.

According to Zhou et al., 2018, nitrite can be converted to unstable HNO_2 under acidic conditions and subsequently decomposed into other nitric oxide species. These reactions can occur at the acidic pH of gastric juice in the stomach (forestomach) of mice (Zhou, R. et al., 2018).

CONCLUSIONS

Prolonged consumption of PAW does not induce methemoglobinemia ($p > 0.05$) and does not affect the health of mice.

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EVALUATION OF KETAMINE - DROPERIDOL ANESTHESIA IN DOGS

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Abstract

The main objective of this study was to observe the influence of anesthesia combined with ketamine and droperidol, compared both in bolus administration and in continuous intravenous infusion, following the effects on cardiac and respiratory function. The experiment was performed on 16 clinically healthy dogs that had previously been pre-anesthetized with acepromazine intramuscularly at a dose of 0.5 mg / kg. The dogs were divided into two groups (A and B). The group A (n = 8) was given the combination ketamine (8 mg / kg) / droperidol (1 mg / kg), intravenously, in a bolus, through the cephalic vein, in a time interval of more than 30 seconds. Group B (n = 8) was given the same combination and through the same vein, but in continuous infusion over a period of approximately 20 minutes. Ketamine and droperidol were mixed in the same syringe shortly before bolus administration. The study was repeated in 6 dogs, using only acepromazine, 0.5 mg / kg, to evaluate the influence of the preanesthetic on the results obtained in the 2 groups A (bolus) and B (infusion). The results of the study showed that the ketamine / droperidol combination should be used with caution in dogs pre-anesthetized with acepromazine, but the effect of anesthesia combined with ketamine and droperidol is better than that of anesthesia only with ketamine.

Key words: anesthesia, ketamine, droperidol

INTRODUCTION

Anesthesia is a common practice in the daily work of a veterinary office for pets. Studies show that there is a very wide range of anesthetic protocols, probably because practitioners are in constant search of a "magic", balanced formula that does not induce side effects and endanger the health of the animal (Farnworth M.J., et al., 2014; Gates M.C., et al., 2020). Unfortunately, there is no single drug that has the characteristics of an ideal anesthetic agent, so we must carefully choose that combination of anesthetics that will help us achieve the desired effects.

Choosing the appropriate protocol for the surgical situation will often allow for lower doses, thus limiting the detrimental influence of these substances on the body's physiological functions. Moreover, many of these combinations of anesthetics work synergistically and thus can correct some of the side effects, which manifest themselves in their separate administration.

With characteristics such as rapid onset and cardio stimulatory properties, the combination of ketamine - droperidol may be ideal for inducing general anesthesia in dogs. Although heart rate and blood pressure may be elevated in non-anesthetized patients, ketamine causes decreases in blood pressure and cardiac output in people anesthetized with halothane (Kurdi M.S. et al., 2014).

Continuous infusion of ketamine and midazolam has been used successfully in human medicine for various investigations (Baillie, R., et al., 1989; Miller A.C. et al., 2011). Intravenous infusion of anesthetic agents for the maintenance of general anesthesia is considered to avoid environmental pollution associated with inhaled anesthetics. Continuous infusion compared to intermittent, injectable administration may result in reduced total need for anesthetic agents, improved intraoperative conditions, and reduced recovery time (Miller A.C. et al., 2011).

In our study, we aimed to evaluate the effectiveness of the anesthesia of the combination of ketamine and droperidol, compared in bolus administration and in continuous intravenous infusion.

MATERIAL AND METHOD

The experiment was performed on 16 dogs of common breed, without owner (9 females and 7 males), with an average weight of 18.1 ± 1.2 kg (M ± DS), clinically healthy. Prior to the experiment, they underwent a rigorous pre-anesthesia physical examination to assess their health. The animals were stopped feeding 12 hours before the study, but the water was ad libitum.

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Pre-anesthesia was performed with acepromazine at a dose of 0.5 mg/kg, intramuscularly, 15 minutes before the general anesthetic combination. Each dog had a branula (i.v. cannula, Novalon 1.25 IN, 20 GA, Becton, Dickinson Co, Sandy) inserted into the cephalic vein. The analysis of blood gases (PaO2 and PaCO2) and pH was performed computerized using the AVL Compact analyzer. The bicarbonate plasma concentration (HCO3-, meq / l) and the basic excess (EB, meq / l) were also calculated.

A non-invasive automatic device (Digital Electronic Blood Pressure Monitor, Model FC 150 D, Tokyo, Japan) was used to measure systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP). Heart rate (beats/minute) was determined by applying a precordial stethoscope. The body temperature was maintained between 37 - 38^0C with the help of Gelpack® (plastic bags with special thermal gel for raising or lowering the body temperature).

The dogs were divided into two groups A and B. The group A (n = 8) was given the combination ketamine (8 mg/kg) - droperidol (1 mg/kg), intravenously, in a bolus, through the cephalic vein a time interval of more than 30 seconds. Group B (n = 8) was administered the same combination through the same vein, but in continuous infusion over a period of approximately 20 minutes. Ketamine and droperidol were mixed in the same syringe shortly before bolus administration. For infusion, the ketamine-droperidol combination was diluted with 0.9% saline shortly before injection.

The study was repeated in 6 dogs, using only the preanesthetic, respectively acepromazine - 0.5 mg / kg, to evaluate the influence of the preanesthetic on the results obtained in the 2 groups A (bolus) and B (infusion).

After performing the first measurements, to obtain the basic data, the ketamine-droperidol combination was administered through the 2 procedures: bolus and infusion. Heart and respiratory rate, SBP (systolic blood pressure), DBP (diastolic blood pressure) and MBP (mean blood pressure) were recorded at minutes 1, 5, 10, 20, 30, 40, 50 and 60 of anesthesia. During the infusion, measurements were also made 5 minutes (-10 min.) and 10 minutes (-5 min.) After administration of the ketamine-droperidol combination. Blood samples were collected at 15, 30, 45, and 60 minutes.

Statistical analysis

Data analysis was performed with the ANOVA test for repeated measurements. When there were significant differences, the comparison of the averages was done using the Tukey test. The Student (t) test was used to compare the averages between the 2 groups: A (bolus) and B (infusion), relative to baseline values to ensure that the study conditions were similar for both groups. A significant level $p < 0.05$ was used.

Ethical implications

The study was conducted in accordance with national and international legal regulations on animal welfare, identification, control and elimination of factors causing physiological and behavioral disorders: Directive EC86 / 609 EU.

RESULTS

Body weight and mean time from induction of anesthesia with acepromazine 0.5 mg/kc to completion of instrumentation and baseline (comparison) were not significantly different between the 2 groups (A and B).

The average time interval from induction to collection of all data necessary to establish baseline values for dogs in the 2 groups A (bolus) and B (infusion) was 63 ± 11 minutes and 52 ± 15 minutes, respectively.

Body temperature decreased significantly over time in dogs in the bolus group (A). The difference in body temperature was significant between the two groups of dogs. Body temperature in group A dogs was on average 1^0C higher than group B temperature.

The hemodynamic effects of bolus and infusion ketamine-droperidol combination are shown in Table 1. In group A (bolus) dogs, changes in heart rate were not significant. In group B dogs (infused), the heart rate decreased after administration of the combination ketamine (8 mg/kg) / droperidol (1 mg/kg), but was not significantly different from baseline after 30 minutes of infusion. anesthesia. The maximum decrease in mean heart rate from baseline was greater in group B dogs, averaging 22 ± 9 beats / minute compared to group A dogs, averaging 7 ± 8 beats / minute.

Systolic, diastolic, and mean blood pressure values decreased significantly immediately after administration of the ketamine-droperidol combination to both groups and were not significantly different from baseline after 30 minutes of anesthesia. The maximum decrease in mean blood pressure compared to baseline values was much higher (38 ± 15 mmHg) in group A

dogs, compared to the value in group B dogs, respectively 18 ± 8 mmHg (Table 2.).

The effects of the ketamine-droperidol combination on pH and blood gas variables are shown in Table 3. Changes in PaCO2, PaO2 and changes in bicarbonate (HCO3-) concentration were not significant. pH and basic excess (EB) values fell below baseline at all-time intervals at which samples were taken and in both groups of dogs, but these changes were significant only in dogs in group B.

In the 6 dogs (3 in group A and 3 in group B), in which the experiment was repeated (after an interval of 14 days) only with acepromazine 0.5 mg/kg, intramusculary. The body temperature decreased significantly over time (during the monitoring period, 60 minutes). Significant increases in heart rate were observed over time and especially at 45 and 60 minutes (times when blood samples were taken) from baseline. Systolic, diastolic and mean blood pressure did not vary significantly over time. Changes in pH, PaCO2, PaO2, HCO3- and EB values were significant. Significant differences were recorded between the control group (n = 6) and group B or group A of dogs at each time interval.

DISCUSSIONS

When ketamine is given intravenously without preanesthesia in healthy dogs, heart rate and blood pressure increase (Haskins, S.C. et al., 1983; Vlerick L. et al., 2020). This response to ketamine is probably an indirect effect. Cardiostimulatory effects can be caused by various physiological mechanisms, including sympathomimetic effects mediated in CNS structures, intraneuronal and extraneuronal inhibition of catecholamine reuptake, and component vagal inhibition of the baroreceptor reflex (Mandsager R., 2003). With the exception of increases in heart rate, diazepam effectively "blocks" the cardiostimulatory properties of ketamine (Haskins, S.C. et al., 1986; Gebremedhin Y., 2018; Gebremedhin Y et al., 2018).

Cardiostimulatory effects may also be evident when higher doses of ketamine are used, and in dogs recovering from general anesthesia with isoflurane or when low doses of it are administered just before isoflurane anesthesia, or when ketamine is administered to humans during halothane or isoflurane anesthesia (Yasuda N. et al., 1991). In contrast, in combination with other anesthetics its action on cardiac properties is different. For example, in the combination of ketamine (28.4 mg / kg, i.v.) / haloperidol (1.42 mg / kg, i.v.), in addition to good muscle relaxation and analgesia, mean blood pressure (MAP) and volume tidal increase significantly (Kumar A., et al., 2001). In the combination of medetomidine (10 μg / kg) / propofol (4 mg / kg) / ketamine (10 mg / gc), administered intravenously, there was depression of cardiac activity (between 6 and 60 minutes), respiratory activity and a decrease in body temperature (Gulanber E., et al., 2001; Ozaydin J., et al., 2001).

In our study, acepromazine (0.5 mg / kg) effectively reduced the cardiorespiratory properties of the ketamine - droperidol combination. Rapid decreases in mean blood pressure in group A (bolus) dogs were probably attributed to decreased cardiac output. In group B (infusion), it appears that a marked decrease in heart rate also contributed to decreases in mean blood pressure, although in several studies ketamine induced positive inotropic effects (Miranda-Cortés A.E., et al., 2020). The results of most studies show that ketamine may have a negative inotropic effect on the myocardium (Hanouz JL., et al., 2004). We cannot say why significant decreases in heart rate are due to infusion of the ketamine / droperidol combination, but not after bolus administration. Probably, large decreases in mean blood pressure at group A (bolus) and any increase associated with sympathetic tone may have served to maintain heart rate.

Maximum decreases in mean blood pressure were much higher in group A than in group B and this indicates that depressant effects may be dose related. Blood pressure was lower in dogs in both groups (A and B) at minute 1 compared to dogs in the control group. This highlights the cardiovascular effects of the ketamine (8 mg/kg) -droperidol (1 mg / kg) combination.

The infusion technique has the advantage of administration control; In any case, it is observed that in group B, the decreases in mean blood pressure were the largest during the first 5 minutes of infusion. This would correspond to the peak concentration of ketamine in the plasma. Although the infusion dosage was lower, cardiovascular depression may be substantial.

From the 10th minute after administration of the ketamine / droperidol combination and until the end of the study, the changes in most variables were parallel to the changes in the control group dogs. Moreover, the rapid redistribution of ketamine may have influenced the immediate return of cardiovascular variables to baseline values. Although only decreases in arterial pH and

basal excess were significant in group B, in general, the decreases were close for dogs in both groups. Although the decreases were clinically insignificant, they indicate a mild metabolic acidosis, which may be the result of inadequate irrigation or oxygenation of the tissues.

The lack of a pressor response can be explained by changes in the activity of the autonomic nervous system during anesthesia. Autonomic activity is attenuated during acepromazine preanesthesia and, as a result, the cardiostimulatory properties of ketamine are not evident when the sympathetic response is blocked. Also, the sympathetic-tonic response to ketamine cannot be observed in critically ill patients due to catecholamine depletion and adrenocortical deposits (Erstad BL., et al., 2016).

There was an increase in mean blood pressure after ketamine administration, probably because the sympathetic (catecholamine) reserves were not depleted.

CONCLUSIONS

The results of the study show that the combination of ketamine (8 mg / kg) / droperidol (1 mg / kg) should be used with caution in dogs pre-anesthetized with acepromazine (0.5 mg / kg).

Infusion is preferable to bolus and induces an anesthetic effect for at least 30 minutes, long enough to perform diagnostic procedures and small-scale surgical procedures (sutures of skin wounds). The recovery of dogs from ketamine-droperidol infusion anesthesia was easy and fast without being accompanied by muscle stiffness, convulsions, vocalization, hypersalivation, etc., phenomena commonly encountered under ketamine anesthesia.

The advantage of preanesthesia with acepromazine (0.5 mg / kg, 15 minutes before the combination) would be to attenuate the activity of the vegetative system, "masking" to some extent the cardiostimulatory effects of ketamine in dogs.

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Table 1.

Mean values (± SD) of cardiovascular indices, bolus versus infusion

Variables	Administration	Basic values	Time								
			-10	-5	1	5	10	20	30	45	60
FC (beats / min.)	Bolus	111±8	ND	ND	107±9	109±7	108±8	108±6	109±6	111±6	109±9
	Perfusion	120±17*	97±14	99±8	103±11	104±16	103±15	104±16	106±13	112±14	117±12
SBP (mmHg)	Bolus	101±11*	ND	ND	55±10*	66±12	76±16*	81±14*	92±8*	97±8*	97±8*
	Perfusion	94±6*	80±4	74±4	73±4	78±4	85±3*	87±8	92±4*	93±8*	96±6*
DBP (mmHg)	Bolus	63±9*	ND	ND	33±4	37±8	49±14	43±4	56±9	58±8	58±9
	Perfusion	57±9*	44±3	40±4	41±3	47±7	54±4	51±8	53±8	53±6	56±9
ABP (mmHg)	Bolus	77±11*	ND	ND	40±7	46±10	58±15	56±5	68±9*	74±10	69±8*
	Perfusion	69±7*	55±6	51±7	53±3	58±3	63±5	63±6	66±8	69±7	72±7
T (0C)	Bolus	39.0±0.1*	ND	ND	38.7±0.7	38.6±0.7	38.4±0.6	38.6±0.6	38.6±0.6	38.7±0.7	38.5±0.8
	Perfusion	39.3±0.8*	38.7±0.1	38.3±3	37.8±0.3	37.7±0.5	37.8±0.5	37.9±0.2	37.5±0.5	37.4±0.4	37.7±0.6

Data are presented as M ± DS. Mean values with * are significantly different. FC- heart rate, SBP - systolic blood pressure, DBP - diastolic blood pressure, ABP - average blood pressure, T (0C) -temperature

Table 2.

Mean (± SD) values of maximal decreases in heart rate and mean blood pressure, bolus versus infusion

Variables	Group A (Bolus I.V.)	Group B (perfusion I.V.)
FC (beats / min.)	6 ± 7	23 ± 11
MAP (mmHg)	38 ± 15	18 ± 8

Data are reported as M ± DS. Significant differences $p < 0,05$. HR – heart rate; MAP – mean arterial pressure

Table 3.

Mean values (± SD) of the acid-base balance variables,
bolus (group A) versus infusion (group B)

Variables	Administration	Basic value	Time (minutes)		
			15	30	45
pH_a (U)	Bolus	7,364±0,065	7,322±0,045	7,304±0,027	7,292±0,033
	Perfusion	7,355±0,061*	7,316±0,043*	7,293±0,030*	7,306±0,049*
P_aCO_2 (mmHg)	Bolus	38,9±6,2	41,8±3,9	41,1±4,5	42,7±4,7
	Perfusion	39,4±4,4	41,8±3,7	41,3±3,8	42,3±3,1
P_aO_2 (mmHg)	Bolus	467,3±48,8	471,5±27,8	474,4±20,0	483,3±20,9
	Perfusion	473,9±42,0	496,9±39,7	490,5±62,7	492,9±40,9
HCO_3^- (meq/l)	Bolus	20,3±1,5	20,5±2,0	21,0±2,7	21,6±1,0
	Perfusion	21,9±1,3	21,3±1,0	20,1±2,6	21,1±1, 1
BE (meq/l)	Bolus	-3,9±1,6	-4,9±1,7	-5,4±1,7	-4,9±0,5
	Perfusion	-2,9±2,5*	-4,5±1,6*	-6,0±2,7*	-4,8±2,1*

Data are presented as M ± DS. Mean values with * significantly different ($p < 0.05$).
pHa - blood pH, PaCO2 - blood pressure CO2, PaO2 - blood pressure O2,
HCO3- bicarbonate concentration, BE - basic excess

AN UPDATE ON ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS AGAINST *MALASSEZIA PACHYDERMATIS*

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Abstract

In recent years, the lipophilic yeast *Malassezia pachydermatis* is considered to be the most significant opportunistic pathogen associated with dermatitis and otitis externa in veterinary medicine. At the same time, various findings have shown the capacity of clinical isolates to acquire azole-resistance, therefore the development of new alternative treatment strategies are highly demanded. In the last decade, plant-based antimicrobials have known a resurrection, and special attention was given to essential oils (EOs). EOs are complex mixtures of small lipophilic molecules, of which one up to three compounds constitute the main phytochemical markers. EOs arose as candidates for the alternative treatment of *Malassezia*-related diseases. This review highlights the antifungal potential of EOs and their bioactive compounds against *M. pachydermatis* based on literature reports (*in vitro* and *in vivo* retrospective studies). A search was conducted using three databases (PubMed, Web of Science, Google Scholar), and all relevant articles from the period 2015-2021 were extracted. The findings showed most of EOs *had* significant *antifungal activity* against *M. pachydermatis*, especially through bioactive compounds such as monoterpenes and sesquiterpenes, on their own or by synergism with conventional antifungal drugs or other compounds, such as fluconazole and Tween 80. EOs with promising antifungal activity against *M. pachydermatis* include winter savory, lemongrass, oregano, cinnamon and oregano. The review emphasizes the importance of Eos as novel antifungal agents. EOs could be considered as an alternative to conventional antifungals, as they act concurrently towards different fungal targets due to their multicomponent nature.

Key words: antifungal, essential oils, *Malassezia pachydermatis*.

INTRODUCTION

In present times, worldwide, thousands of plant species are being used to obtain modern medication and at the same time, being used in traditional medicine.

Increasingly aggressive microorganisms, with a multi-drug resistant profile, urged the reduction of chemicals being used as antimicrobial agents and focused medical professionals on substances derived from plants, such as hydro-alcoholic extracts or essential oils.

The versatility of such substances is enormous as the same plant can provide a pool of substances with a very broad spectrum of action due to their different chemical structure. The term "Essential Oil" (EO) was coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim. Approximately 17,000 plant species produce essential oils. These are belonging mainly to a few families, and the most representative are *Lamiaceae*, *Asteraceae*, *Myrtaceae*, and *Lauraceae* (*Bruneton, 1999)*. Currently, about 4000 EOs are known, the number growing exponentially every day. Most of these EOs are being used in the food and beauty industry as flavours and fragrances. Plant EOs represent a complex mixture of compounds, are known for their antiseptic and medicinal properties (sedative, analgesic, anti-spastic, anti-carcinogenic) and, furthermore, due to their antimicrobial and antioxidant potential, are used as natural additives in foods and food products. Many thousands of compounds belonging to the family of terpenes have so far been identified in essential oils, mainly of terpenoidic nature *(Bilia et al., 2014)*

Terpenoids represent groups of hydrocarbons which have as base structure the isoprene (C_5H_8). They represent the largest group of phytochemicals with the highest antimicrobial potential. They are classified in 8 categories based on the number and structure of the isoprene units. Studies from 2017 have showed that 67% of the terpenoids that exhibit bioactivity are represented by monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$). As for the antimicrobial activity of terpenoids, the mechanism of action is not fully understood, but recent studies report that the majority of terpenoids inhibit two crucial survival processes of microorganisms: oxidative

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phosphorylation and oxygen absorption. Antifungal components can also lead to dysfunction of fungal mitochondria at the same time disrupting the formation of the fungal cell membranes.

Malassezia spp. represents a group of lipid-dependent fungi, commensals on the skin of humans and animals. Through mechanisms still incompletely elucidated, they become pathogenic and are involved in a variety of skin conditions, both in human medicine - pityriasis versicolor (*M. furfur)* and veterinary medicine - complications in atopic dermatitis *(M. pachydermatis).*

Despite the extraordinary advancement of mycological knowledge in the last two decades in the study of these yeasts, the pathogenetic mechanism and virulence factors are not fully clarified. The ability of this group of microorganisms to colonize and infect is determined by complex interactions between the fungal cell and the host, through virulence factors. Current *in vitro* and in *vivo* studies mention the presence of *M. pachydermatis* strains with increased tolerance to azoles, due to point mutations in the ERG11 gene.

Recent studies *(Chiavassa et al., 2014, Watanabe et all., 2014)* have reported the sporadic presence of *M. pachydermatis* strains with increased tolerance to azoles in vitro. In 2019, Angileri et al. isolated a strain from a Toy-Poodle that did not respond to azole treatment, which resulted in a 7x higher MIC of itraconazole. In 2018, Rui Kano et al. isolated the first multi-resistant azole strain with mutations in the ERG11 gene, which has a MIC of 320 μg / ml for itraconazole and over 32 μg / ml for ketoconazole. This justifies the importance of alternative therapeutic research, which has caused an increased interest in studying the antifungal potential of EOs.

In the field of veterinary medicine, EOs have been used as prevention against ectoparasites, they have shown a positive effect in atopic dermatitis, chronic dermatitis, pyoderma and malodor in canines. EOs can also be used as ingredients in mouth rinses for their antimicrobial properties or in the treatment of abscesses.

This review highlights the antifungal potential of EOs and their bioactive compounds against *M. pachydermatis* based on literature (*in vitro* and *in vivo* retrospective studies).

MATERIALS AND METHODS

A search was conducted using three databases (PubMed, Web of Science, Google Scholar), and all relevant articles from the period 2015-2021 were extracted.

RESULTS AND DISCUSSION

In vitro tests

Authors reported different assays to evaluate the antifungal properties. Broth microdilution assay was the most used, followed by the agar disk diffusion test and vapor phase methods. This review focused on species with implication in veterinary medicine, but some authors included in their studies species involved in human pathology. The strains tested in the studies are represented by clinical isolates. All of the authors cited in this review evaluated the antimycotic activity of different essential oils against *M. pachydermatis*. The literature related to the last five years shows a great variety of essential oils originating from different plant genera (i.e. *Thymus, Artemisia, Malaleuca, Cinnamomun, Ocimum, Zataria, Rosmarinus, Origanum, Syzigium, Foenicolum, Thapsia, Tachyspermum, Myrtus*). Different assays were used to evaluate the antifungal proprieties. Agar diffusion test was the most used, followed by broth microdilution assay.

Váczi, P. et al. tested the antifungal activity of 14 selected EOs at 3 different concentrations: 0.5%, 5% and 30% against 18 clinical isolates and one reference strain of *M. pachydermatis*. The isolates were collected from canine patients diagnosed with otitis externa. Out of the tested EOs, clove, cinnamon and oregano showed a 100% antifungal efficacy at 30% concentration, whereas at the concentration of 5% the efficacy was less significant (38%, 33% and 5%, respectively). The main active compound in the previously mentioned EOs is represented by eugenol (77%). Satureja inhibited the growth at 30% concentration with an efficacy of only 16%. The remaining 10 EOs tested did not exhibit any inhibition zone greater or equal to 15 mm which was defined in the methodology used, therefore they were considered ineffective. Regarding all the EOs, the 0.5% concentration was not effective inhibiting the growth of *M. pachydermatis*.

Table 1

Effectiveness of the EOs against *M. pachydermatis* according to Váczi, P. et al.

EO	5% concentration			30% concentration		
	Isolates		Reference strain	Isolates		Reference strain
	Inhibition zones (mm)	% of efficacy (no of sensitive strains/no of samples tested)	Inhibition zones (mm)	Inhibition zones (mm)	% of efficacy (no of sensitive strains/no of samples tested)	Inhibition zones (mm)
Clove	16,94	38% (7/18)	18	41,67	100% (18/18)	44
Cinnamon	15,44	33% (6/18)	15	40,14	100% (18/18)	50
Oregano	13	5	14	38,8	100%(18/18)	52
Satureja	7,49	0	0	23,26	16% (3/18)	12
Cedar	3,22	0	8	11,94	0	12
Chamomile	3,61	0	6	11,17	0	12
Bergamot	0	0	0	8,44	0	10
Lavender	0	0	0	6,55	0	8
Grapefruit	0	0	0	10,78	0	10
Sage	0	0	0	10,06	0	14
Tea tree	0	0	0	8,44	0	10
Juniper	0	0	0	5,55	0	8
Pine	0	0	0	4,44	0	6
Yarrow	0	0	0	0	0	0

Table 2

Effectiveness of the EOs against M. pachydermatis according to Bismarck et al.

EO	Inhibition zone radius (median)			
	Aromatogram	Vapour assay	EO 20% solution	EO 10% solution
Winter savory	>40	>40	10.75	4.5
Lemon grass	>40	>40	19.5	8
Rose geranium	>40	>40	7	0
Oregano	>40	>40	14.5	6
Palmarosa	>40	>40	12.5	6.75
Indian melissa	>40	>40	7	0
Thyme thymol 19%	37.5	>40	0	n.t.
Cinnamon leaf	26	24	9	4
Clove	25	22.5	8	5
Thyme	19.75	21.5	0	n.t.
Coriander seed	19	17	0	n.t.
Thyme linalool	16.5	13	0	n.t.
Manuka	15	10.5	5.5	0
Tea tree	10	7.5	0	n.t.
Lavandin super	9	9.5	0	n.t.
Fennel	8.75	0	0	n.t.
Lavender fine	8	7.25	0	n.t.
Lemon	7.75	4.5	0	n.t.
Clary sage	7.5	5.5	0	n.t.
Angelica root	7.5	0	0	n.t.
Ravintsara	6.75	0	0	n.t.
Neroli	0	0	0	n.t.

n.t – not tested;

Bismark et al., tested 22 EOs on 15 canine clinical isolates using two different assays – Agar disk diffusion with 20%, respectively 10% EO concentration and vapor assay. Out of the 22 EOs tested, winter savory, lemon grass, oregano, palmarosa, and cinnamon leaf oil showed excellent in vitro activity. The activity of antifungal agents was tested simultaneously using agar disc diffusion assay. The efficacy of the EOs was classified based on the inhibition radius as follows: not sensitive <8mm, slightly sensitive 8-13.9mm, moderately sensitive 14.0-19.9 mm, very sensitive >20mm, extremely sensitive if no growth.

Ebani et al., conducted the first study that tested simultaneously the efficacy of EOs against *Staphylococcus spp*. and *Malassezia spp*. strains isolated from canine patients. The antifungal activity was conducted by using Agar Disk Diffusion Method.

Five clinical isolates of *M. pachydermatis* were tested, these strains were isolated from the skin of dogs diagnosed with atopic dermatitis. The antifungal activity of selected EOs was assessed by microdilution method, diluted in dimethyl sulfoxide (DMSO, Oxoid Ltd.), at concentrations of 5%, 4.5%, 4%, 3.5%, 3%, 2.5%, 2%, 1.5%, 1%, 0.75%, and 0.5%. MIC was established as the lowest concentration of EO where no fungal growth was yielded. *Commiphora myrrha* and *Litsea cubeba* were not effective at 5% dilution. *Aloysia tryphilla* was the most active with MICs of 0.87 and 1.03 mg/mL, followed by *S. montana* with MIC of 1.8 mg/mL and *Cinnamomum zeylanicum* with 3.06 and 4.08 mg/mL. *Malassezia yeasts* showed a marked variability in their susceptibility to EOs. For these reasons, a sensitivity assay of the fungal isolates is recommended, as suggested by Bismarck et al.

Table 3

MIC values of the tested EOs expressed in mg/mL against selected *M. pachydermatis* isolates according to Ebani et al.

EO	UNIT	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
Aloysia triphylla	mg/mL	1.03	0.87	1.03	0.87	1.03
Cinnamomum zeylanicum	mg/mL	3.06	3.06	4.08	3.06	4.08
Commiphora myrrha	mg/mL	n.e.	n.e.	n.e.	n.e.	n.e.
Cymbopogon citratus	mg/mL	7.13	7.13	7.13	7.13	7.13
Litsea cubeba	mg/mL	n.e.	n.e.	n.e.	n.e.	n.e.
Melissa officinalis	mg/mL	3.55	3.55	3.55	3.55	2.66
Origanum vulgare	mg/mL	7.73	7.73	6.76	7.73	6.76
Satureja montana	mg/mL	1.8	1.8	1.8	1.8	1.8
Thymus vulgaris	mg/mL	8.7	8.7	7.73	7.73	7.73
Cloramphenicol	µg/mL	0.02	0.02	0.02	0.02	0.02

Aiemsaard J. et al. in 2020, evaluated the antifungal activity of the essential oil in the betel vine against 17 strains of *M. pachydermatis* isolated from lesions of canine dermatitis, using the microdilution assay and time kill assay. The predominant EO in the betel vine was eugenol (32.82%). The results were promising, the EO having a high antifungal activity against all tested isolates, with MIC and MFC values equal to 0.66 – 1.13 µg/ml. The time kill assay showed that the highest activity was achieved by 8 times the MIC, which eradicated more than 99.9% of the microorganisms within 30 minutes, followed by 4 times the MIC, which eradicated tested microorganisms by 90% in less than 30 minutes. In a different study, in 2019, the same authors tested clove EO and eugenol on 17 strains of *M. pachydermatis* isolated from canine patients and found no significant differences between the two EOs MPICs, MPFCs, MBICs and MBECs, suggesting that eugenol was the major component of the clove EO, which justifiably led the authors to continue with the aforementioned study. On the other hand, they found that the yeast biofilms were 2 times more tolerant to the EOs tested, a much lower figure than previously reported in 2013 by Figueredo et al.

Table 4

MPIC50 and MPFC50 for 17 planktonic M. pachydermatis isolates according to Aiemsaard J. et al.

Antifungal agent	MPIC50 (mg/mL)	MPFC50 (mg/mL)
Clove EO	0.156	0.312
Eugenol	0.156	0.312
Ketoconazole	0.019	0.038

$MPIC_{50}$ – minimum planktonic inhibitory concentration for 50% of tested isolates; $MPFC_{50}$ - minimum planktonic fungicidal concentration for 50% of tested isolates.

Table 5

MBIC50 and MBEC50 for biofilms of 6 M. pachydermatis isolates according to Aiemsaard J. et al

Antifungal agent	MBIC50 (mg/mL)	MBEC50 (mg/mL)
Clove EO	0.312	0.624
Eugenol	0.312	0.624
Ketoconazole	0.038	0.076

MBIC50 – minimum inhibitory concentration for 50% of tested isolates; MBEC50 – minimum biofilm eradication concentration for 50% of tested isolates.

Jowenna Xiao Feng Sim et al. tested the in vitro efficacy of the main phenolic constituents of oregano oil, thyme oil against 20 *M. pachydermatis* clinical isolates associated with canine otitis externa. The antimicrobial susceptibility testing was evaluated by using broth microdilution with spot-planting assay to determine the MIC, MBC and MFC. To confirm the fungicidal activity of the EOs, a time-kill kinetics assay was performed. Oregano oil (carvacrol) and thyme oil (thymol) showed promising antifungal activity against *M. pachydermatis* isolates. They were more sensitive to oregano oil (MIC90 = 0.06%; 563–585 lg/mL), in detriment to thyme oil (MIC90 = 0.125%; 1,146 lg/mL). Additionally, *M. pachydermatis* was reported to have the same MFC values as their MIC values.

Table 6

MIC50, MIC90, MFC50 and MFC90 of tested EOs on 20 isolated from dogs with Malassezia otitis externa according to Jowenna Xiao Feng Sim et al.

Concentration, % values (μg/mL)				
Value	**Oregano**	**Carvacrol**	**Thyme**	**Thymol**
MIC_{50}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
MIC_{90}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
MFC_{50}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
MFC_{90}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)

In 2015, A.R. Khosravi et al. evaluated the antifungal efficacy of medicinal EOs on 84 strains isolated from canine patients, from various parts of the body (ear, mouth, interdigital, groin). The authors collected plants from different regions of Iran, specifically *Zataria multiflora*, *Thymus kotschyanus*, *Mentha spicata*, *Artemisia sieberi*, *Rosmarinus officinalis* and *Heracleum persicum*. EOs were isolated by water distillation, according to the European Pharmacopoeia. The MICs of the 6 EOs, were evaluated by broth microdilution method. Out of the tested oils, two of them were more active than the other: *Z. multiflora* (MIC90 value: 60 μg/mL) and *A. sieberi* (MIC90 value: 80 μg/mL), having the strongest antifungal proprieties against *M. pachydermatis* and other species, containing a high percentage of phenolic compounds, such as thymol and carvacrol.

Table 7

MIC90 of some essential oils against M. pachydermatis, according to A.R. Khosravi et al.

Agent	MIC90 (μg/mL)
Zataria multiflora	60
Thymus kotschyanus	100
Mentha spicata	150
Artemisia sieberi	80
Rosmarinus officinalis	360
Heracleum persicum	580

In vivo studies

Unfortunately, in vivo studies in the veterinary field at this time are scarce. From the aforementioned databases, only two such studies were retrieved.

Nardoni S. et al., in 2017, tested in vivo, five mixture of essential oils that were obtained from plants found in the Mediterranean area. The EOs themselves showed promising results in previously reported in vitro studies: *Citrus paradisi*, *Salvia sclarea*, *Ocimum basilicum*, *Rosmarinus officinalis*, *Citrus limon*, *Anthemis nobilis*, *Lavandula hybrida* and *Thymus vulgaris*.

The study centered on testing 25 atopic dogs, diagnosed with Malassezia otitis externa, by treating them once daily for 2 weeks. The mixture comprised by C. limon 1%, S. sclarea 0,5%, R. officinalis 1%, A. nobilis 0,5% showed the best results in all treated dogs. There was a complete clinical resolution of symptoms, although the number of blastopores did not decrease. All of the mixtures were tested comparatively with ketoconazole with a MIC of < 0.03 μg/ml−1. Individually, out of all the EOs tested, *T. vulgaris* achieved the lowest MIC (0.05%). The authors mentioned caution when using some EOs topically as some of them can contain irritant compounds (i.e. *O. basilicum*, citrale, limonene) and also discourage the use of high EOs concentrations, especially in atopic patients, as it can cause skin adverse effects. Their concern is based on the fact that 2 patients had adverse effects to one of the mixtures containing 2% Citrus spp. The aforementioned mixture, with the highest grade of the success contains EOs characterized by eudermic action (S. sclarea and R. officinalis).

Rita C.S.M. Neves et al., in 2019, conducted a study on 28 dogs with clinical signs

of otitis externa, subsequently diagnosed as being fungal otitis externa, identifying *M. pachydermatis* as the primary pathogen. The aim of the study was to compare the effects of tea tree EO with common antifungal topic treatments. The authors used a 5% tea tree EO lotion in the right ears, while using a 0.15% nystatin lotion in all the left ears, as positive control. They concluded there was no significant difference between the two treatments. Not all dogs treated with tea tree EO have healed completely in the 14 days trial, but a significant improvement of the clinical and cytological findings was obvious on all patients, less inflammation, pruritus, discharge and a lower quantification of microorganisms and inflammatory cells.

CONCLUSIONS

Chemical drugs are being associated with the rapid emergence of multi-drug resistant microorganisms while traditional medicinal plants represent a very important reservoir of active substances that can be used in as aid in treatment of infectious diseases, due to their already known antimicrobial effects. The studies reviewed in this summary show that EOs have a great antimicrobial potential and should be used alone, or in addition with antifungal agents.

Due to different study designs, test methods and of course, the EOs used in the assays, a comparison of results is not always possible. Testing more than one isolate reveals different susceptibility of *M. pachydermatis* to EOs, indicating that an EO might help on one strain isolated from a specific patient, but might not be the best option for a different individual. More in vivo studies need to be conducted to evaluate the risk-benefit ratio of EOs in treating Malassezia related infections.

Further studies to determine the suitable concentration and formulation of EOs and in vivo testing of the antifungal efficacy against *M. pachydermatis* infections are required.

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