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CONTENTS

IMPACT OF RECENT AND FUTURE CLIMATE CHANGE ON VECTOR-BORNE DISEASES: VIRUSES ANALYSES

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Abstract

Climate directly impacts health through climatic extremes, air quality, sea-level rise, and multifaceted influences on food production systems and water resources. Climate also affects infectious diseases, which have played a significant role in human history, impacting the rise and fall of civilizations and facilitating the conquest of new territories. This review highlights significant regional changes in vector and pathogen distribution , changes that have been anticipated by scientists worldwide . Further future changes are likely if we fail to mitigate and adapt to climate change. Many key factors affect the spread and severity of human diseases , including mobility of people , animals , and goods ; control measures in place; availability of effective drugs; quality of public health services; human behavior; and political stability and conflicts.

Keywords: vector-borne viruses, climate change

Stade of art

Climate change is considered one of the greatest threats to human health by the World Health Organization. The rate of global warming which has occurred during recent decades has been unprecedented over the past millennium, and there is consensus in the scientific community that the cause is increasing anthropogenic emissions of greenhouse gases. Climate change directly impacts health through long-term changes in rainfall and temperature, climatic extremes (heatwaves, hurricanes, and flash floods), air quality, sea-level rise in low- land coastal regions, and multifaceted influences on food production systems and water resources . Climate has a direct impact on the dynamics of a subset of infectious diseases, including vector-borne diseases (VBDs), some water-borne diseases such as cholera, and other soil-borne and food-borne pathogens. Climate also has multiple indirect effects through socioeconomic factors; as one example, flooding can hamper disease control measures in place, including vector control.

In March 2022, the report delivered by intergovernmental panel on climate change (IPCC) warned that without swift climate action we would see an escalation of infectious diseases. Infectious diseases will spread to new regions, surge in areas where they were previously under control and new infectious diseases could infects humans from ‘spill over' from animals. In Europe, several arthropod-borne viruses (arboviruses) are of concern to public authorities, such as the chikungunya virus (CHIKV), the dengue fever virus (DENV), the zika virus (ZIKV) or the West Nile virus (WNV), all 4 transmitted by mosquitoes, but also the tick-borne encephalitis virus (TBEV) and the Crimean-Congo hemorrhagic fever virus (CCHFV) both transmitted by ticks. For some of these arboviruses (such as WNV, DENV and TBEV), several hundred human cases (imported or indigenous) are detected each year in Romania. For others (such as CCHFV, ZIKV, CHIKV), the risk of emergence is taken very seriously and should be actively monitored. The European Union has ranked WNV as high priority and CCHF-virus (CCHFV) has been one of the eight priority emergent pathogens for the last 3 years by the

World Health Organization (WHO), requiring urgent attention in research, development and innovation because of their expansion and epidemic potential in the near future.

The transmission cycles of vector-borne diseases are sensitive to climatic factors, but disease risks are also affected by factors such as land use, vector control, human behaviour, population movements and public health capacities. Climate change is regarded as the principal factor behind the observed move of the tick species *Ixodes ricinus* — the vector of Lyme borreliosis and tick-borne encephalitis in Europe — to higher latitudes and altitudes. A similar phenomenon is observed for *Hyalomma marginatum*, the main vector of the Crimean-Congo Hemorrhagic fever (CCHF). Climate change is projected to lead to further northwards and upwards shifts in the distribution of *Ixodes ricinus* and *Hyalomma marginatum*.

It is generally suspected that climate change has played (and will continue to play) a role in the expansion of other disease vectors, notably the Asian tiger mosquito (*Aedes albopictus*), which can disseminate several diseases including dengue, chikungunya and Zika, and phlebotomus sandfly species, which transmit leishmaniasis.

Vector-borne diseases represent 17% of infectious diseases causing 700,000 deaths each year and economic losses of around one billion euros (WHO, 2020). VMs are caused by infectious agents (mainly viruses but also bacteria and parasites) transmitted to vertebrates by the bite of a hematophagous arthropod vector. Among them, mosquitoes are the first vectors of pathogens in human health while ticks are the first vectors in animal health (and second in human health) ([1].

In Europe, several arboviruses (arthropod-borne virus) are of concern to public authorities, such as the chikungunya virus (CHIKV), the dengue fever virus (DENV), the zika virus (ZIKV) or the West Nile virus (West-Nile virus, WNV), all 4 transmitted by mosquitoes, but also the tick-borne encephalitis virus (TBEV) and the Crimean-Congo hemorrhagic fever virus (CCHFV) both transmitted by ticks. For some of these arboviruses (such as WN, DENV and TBEV), several hundred human cases (imported or indigenous) are detected each year in France, Romania and Moldova. For others (such as CCHFV, ZIKV, CHIKV), the risk of emergence is taken very seriously and should be actively monitored.

Ticks are a major risk factor for animal and human health by transmitting the greatest variety of pathogens (bacteria, parasites and viruses) compared to other groups of arthropod vectors ([2, 3]). They affect livestock as well as humans, as well as pets and leisure animals. The abundance and activity of ticks are very dependent on the movements of wild animals and changes in biotopes. In fact, on a global scale, the intensification of human and animal movements as well as environmental changes are among the main factors for the emergence and/or extension of the range of many pathogens. transmitted by ticks, often zoonotic and for some epizootic. Among the pathogens transmitted by ticks, there are bacteria including the agents responsible for borreliosis, rickettsiosis, anaplasmosis, bartonellosis, ehrliochiosis, but also parasites responsible for babesiosis and theileriosis, and finally viruses responsible for encephalitis and/or hemorrhagic fever. At European level, the two main diseases are ([4]) Lyme borreliosis with an estimate of nearly 65,000 new cases diagnosed each year (and 300,000 cases annually in the USA) ([5]), tick-borne encephalitis which represents the most important neuroinvasive disease transmitted by ticks in Europe and Asia with several thousand human cases per year, including a hundred in France since 1968, with a recent increase in their incidence (around 30 cases per year). The diseases induced are for some of them very difficult to diagnose and the control and prevention strategies remain very complicated to implement since they require the disruption of a complex chain of transmission involving vertebrate hosts and ticks that interact in a constantly changing environment.

Viral vector borne diseases

CCHF is a widespread tick-borne viral disease caused by an *Orthonairovirus* of the *Nairoviida*e family. CCHFV has been considered to be one of the eight priority emergent pathogens for the last 3 years by the World Health Organization (WHO), requiring urgent attention in research, development and innovation because of its epidemic potential in the near future.

While infected animals are usually asymptomatic, humans can develop a serious infection. In humans, CCHFV infection can lead to a severe, life-threating disease characterized by hemodynamic instability, hepatic injury, and neurological disorders, with a worldwide lethality rate from 5 to 40%.

This infection is a public health concern given the increase in its distribution area, particularly in Europe, and more particularly in Turkey and the Balkans. Currently, less than 20,000 cases of infection with this virus have been confirmed worldwide. In Turkey, no cases had been identified before 2002; since then, more than 9,700 cases have been diagnosed [6]). The CCHFV

was ultimately found to be endemic in more than 50 countries across Africa, Asia, Europe, and the Middle East.

Like many other viruses, the CCHFV circulates on several continents, depending on the distribution of its vector, the tick, and its different hosts which can be sedentary or migratory. With global warming, the distribution areas of its vector seems to change, thus modifying the circulation of the virus, with the effect of increasing the risk of emergence in new geographical areas ([7]). Climate changes of recent decades have recently led to a rise in the distribution of this virus.

West Nile virus (WNV), Usutu virus (USUV), and the tick-borne encephalitis virus (TBEV) are all arboviruses belonging to *Flaviviridae* family, characterized by vectorial transmission and sometimes associated with neuroinvasive infections. The circulation of these viruses is considered endemic in some parts of Europe with cases reported in many countries. Hematophagous arthropods such as mosquitoes (WNV, USUV) and ticks (TBEV) transmit the virus among hosts. Romania has a long history regarding the circulation of the WNV, the disease being first reported in 1955 [29]. In addition, during 1996, Romania also recorded the most important human outbreak reported in Europe at the time, in the south-eastern region of the country. Of the 393 human cases recorded, 352 were severe forms of meningoencephalitis [30]. A continued transmission was observed during the next years but with a lower number of clinical infections [31]. Another notable outbreak occurred during 2010, with 52 confirmed cases and a 10% mortality rate. Although most infections were located in the southern part of the country, new cases have also been reported inside the arch of the Carpathian Mountains [29]. Moreover, during 2016, another disease outbreak was recorded in Romania, this time registering 93 neurological human cases [32]. To date, the most severe outbreak registered in Europe in recent years took place in 2018, when Romania registered 277 clinical cases and 43 deaths out of a total of 2083 human clinical infections at the European level. A decrease in infections was observed in the following years[33-35]. Since 1997, a passive surveillance system has been implemented in Romania. Every year, from June to November, blood serum and cerebrospinal fluid from suspect cases of human WNV-associated central nervous system infections in patients over the age of 15 years old are screened using IgM WNV enzyme-linked immunosorbent assay (ELISA). A 28-day quarantine period is mandatory for all blood donors from localities where human cases have been detected. Nevertheless, a significant number of human viral encephalitis cases remain unconfirmed for WNV and are recorded as "viral encephalitis with unknown etiology", as the current legislation does not require further confirmatory tests for other arthropod-borne arboviruses. As a consequence, the blood donors are not screened for other viruses although some, i.e., Toscana[36]and Usutu, were recently reported in Romania.

USUV, a member of the Japanese encephalitis serocomplex, is phylogenetically close to WNV [37-39]. USUV has spread to a large part of the European continent over the two decades, mainly leading to substantial avian mortalities with a significant recrudescence of bird infections recorded throughout Europe within the last few years[40]. In Europe, USUV was first reported in Austria and was associated with high mortality among blackbirds (*Turdus merula*)[41]. This event was followed by a retrospective study in Italy on bird tissue samples stored from 1996, which subsequently tested positive for USUV by molecular techniques[42]. Later, the virus was identified in mosquitoes and different vertebrate hosts in several European countries[43, 44]. USUV infection in humans is considered to be most often asymptomatic or to cause mild clinical signs[40]. The first human neuroinvasive infection in Europe was registered in 2009 in Italy in a patient with meningoencephalitis symptoms [45] followed by other reports[46]. Seroconversion in healthy blood donors was also registered[47]. In Romania, the first serological evidence of the presence of USUV was documented in a domestic dog, but its presence in humans has not yet been demonstrated.

TBEV is endemic in many European countries including Romania, being the most important neuroinvasive arbovirus vectored by ticks[48, 49]. Information on the tick- borne encephalitis (TBE) epidemiology in Romania is scarce and partly outdated. The most important outbreak in humans was recorded in 1999, when 38 infections were recorded, raw goat dairy products being incriminated as the source of infection. Seroprevalence rates varied between 0.0% and 41.5% in humans and between 0.0% and 27.7% in livestock [50]. Since 2008, TBE has been passively monitored in 11 north-western and central counties considered at risk out of the 41 counties of Romania. TBE is also notifiable at the EU level since 2012[51].

CONCLUSIONS

There is a wealth of evidence that recent climate change has already affected pathogen–vector–host systems, in particular over temperate, peri-Arctic and Artic areas and high altitude

regions in the tropics. There are now many examples of the early impacts of climate change on animal VBD bur- den, while the most severe VBD outbreaks affect- ing humans tend to be affected by a myriad of complex socioeconomic factors and climate. This review demonstrates that the spread of vectors and the pathogens they transmit worldwide has been anticipated by scientists.

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CRIMEAN-CONGO HEMORRHAGIC FEVER: A FUTURE HEALTH ISSUE IN FRANCE? WHAT ABOUT ROMANIA?

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Abstract

Crimean Congo Hemorrhagic Fever Virus (CCHFV) is the etiological agent of a severe hemorrhagic fever affecting Africa, Asia and southern Europe. In recent decades, climate change has led to an increase in the distribution range of this virus. Little scientific data is yet available on the interactions with its vector, the tick, or on its biology. However, the confirmed presence of human infections in Spain and positive serologies in Corsican livestock could well focus attention on this pathogen. This review takes stock of developments in eco-epidemiological knowledge of this virus, particularly in Europe and especially in France. What about Romania ?

Keywords: Crimean-Congo hemorragic fever, virus, emergent disease

Introduction

Crimean Congo Hemorrhagic Fever Virus (CCHFV) is the etiological agent of a severe hemorrhagic fever affecting Africa, Asia and southern Europe. In recent decades, climate change has led to an increase in the distribution range of this virus. As yet, little scientific data is available on interactions with its vector, the tick, or on its biology. However, the confirmed presence of human infections in Spain and positive serologies in Corsican livestock could well focus attention on this pathogen. This review takes stock of developments in eco-epiological knowledge of this virus, particularly in Europe and especially in France.

The viral cycle from an ecological standpoint

The distribution of the CCHFV virus overlaps with the location of various species of hard ticks in the Ixodidae family (notably the Hyalomma genus), its natural reservoirs. In the wild, *Hyalomma* tick larvae preferentially infect small mammals (rodents and lagomorphs) or birds, where they transform into nymphs. The nymphs then detach from their host and moult into adults, which seek out a new host, usually a large mammal. These large mammals thus serve as amplifying hosts without showing any symptoms. Infected female ticks lay eggs that carry the virus (transovarial transmission), giving rise to a new generation of infected vectors. Recently, reptiles have been added to the list of species playing a role in the circulation of CCHFV, with its discovery in the blood of Testudo turtles and in the *H. aegyptiacum* ticks that parasitize them. The disease is mainly transmitted to humans by tick bites, but it can also be transmitted through contact with biological fluids from infected animals, hence the high risk for livestock farmers, veterinarians, nursing staff and slaughterhouse personnel.

Most of the tick species that transmit the CCHFV virus belong to the hard tick family, the Ixodidae. These ticks are vectors of numerous viral and bacterial pathogens (causing tick-borne encephalitis, borreliosis, rickettsiosis, etc.). The CCHFV virus has been isolated mainly in Eurasia and Africa, from various ticks belonging mainly to the genus *Hyalomma*, but also from ticks of the genus *Amblyomma*, *Rhipicephalus* (and ssp boophilus*)*, *Ixodes*, *Haemaphysalis* or

Dermacentor. Ticks of the *Hyalomma* genus (as well as those of the Amblyomma genus) are known for their specific active hunting behaviour. These different Ixodes are characterized by feeding on one, two or three hosts during their lifetime. Ticks that feed on a single host during their development (e.g. Rhipicephalus of the Boophilus subgenus) represent only a small proportion of ticks associated with CCHFV. Ticks with two or three hosts, on the other hand, can transmit the virus to different branches of mammals. Unlike the Ixodidae family, ticks of the *Argasidae* family, known as soft ticks, are described as multi-host. They feed on 5 to 20 different hosts during the different stages of their life. Very few studies mention the presence of CCHFV in these species (only in the genera *Argas* and *Ornithodoros*), and attempts to infect these ticks with the virus in the laboratory have been unsuccessful. The short duration of the blood meal in these species seems to be associated with a lower capacity to transmit the virus, compared with Ixodidae, whose meal can last several days. In infected ticks, the virus is mainly found in the salivary glands and reproductive organs it is through saliva that the virus is transmitted. Several other viruses are currently under development, and should in future enable the virus to be transmitted to the parasitized animal.

Pathophysiology of infection

While infected animals are generally asymptomatic, humans can develop severe infection. The disease resulting from CCHFV infection has four phases: an incubation phase, a pre-haemorrhagic phase, a haemorrhagic phase and a convalescence phase, the duration and associated symptoms of which can vary considerably. The incubation period after a tick bite can last from one to three days, and in some cases more than a fortnight; this is linked to the mode of transmission of the disease (by the tick itself or by contaminated fluids). The pre-haemorrhagic phase is characterized by a sudden rise in fever, dizziness, headaches, photophobia and back and stomach pains. The hemorrhagic phase, which develops 3 to 6 days after the onset of the disease, is associated with petechiae1 and ecchymosis, blood in urine and feces, and external bleeding (nose, gums, skin, etc.). In the most severe cases, cerebral haemorrhage and massive liver necrosis can occur. These are associated with a poor prognosis.

In humans, hemorrhages are the consequence of endothelial cell fragility, due to infection, but also of a cascade of host-induced mechanisms in response to the virus. The resulting deregulation of hemostasis stems in particular from the induction of pro-inflammatory cytokine secretion and disseminated intravascular coagulation. The mortality rate of CCHF after natural infection varies between 5% and 40%. Nosocomial viral infections have also been observed. These have higher mortality rates, no doubt due to the greater viral inoculum involved. In this case, the virus is transmitted via contact between a contaminated patient's biological fluids and the caregiver's mucous membranes. This type of transmission has also been observed following veterinary care or in slaughterhouses.

Survivors undergo a long convalescence phase, starting 15 to 20 days after the onset of the disease. At present, only ribavirin is used to treat the infection. This nucleotide analogue appears to have some activity when used in the early stages of infection, although its efficacy remains debatable. Very recently, an American team demonstrated that the use of antibodies specific to GP38 (a soluble non-structural viral glycoprotein) could protect animals against the virus. GP38 is little studied, and this first discovery opens the way to new fields of research, both for understanding the virus and developing new treatments. A single vaccine is available, but only in Bulgaria.

Epidemiology du virus

In Tajikistan, texts dating back to the 12th century refer to a disease with haemorrhagic symptoms similar to those of CCHF. But it was in 1944, during the Second World War, in the Crimea, that the virus was first identified as the etiological agent of a contagious hemorrhagic fever that caused an epidemic. The virus responsible was isolated and named "Crimean haemorrhagic fever virus". In 1969, it turned out that this virus was in fact identical to the one responsible for Congo fever, which had been isolated.

Of Congo fever, isolated in 1956, hence the name "Crimean-Congo hemorrhagic fever virus" (CCHFV for the virus and CCHF for the disease). The CCHFV virus eventually proved endemic in over 30 countries across Africa, Asia, Europe and the Middle East.

This infection is a public health concern in view of its increasing range, particularly in Europe, and more specifically in Turkey and the Balkans. Currently, less than 20,000 cases of infection with this virus have been confirmed worldwide. In Turkey, no cases were identified before 2002; since then, over 9,700 cases have been diagnosed.
The geographical distribution of CCHFV coincides with that of ticks of the genus Hyalomma, notably H. marginatum, its main vector in Europe. These

ticks can be found sporadically as far north as Germany and Sweden, transported during avian migrations or livestock movements. The local climate in these regions did not allow these tick species to establish themselves permanently, but the recent discovery in Germany of ticks of the Hyalomma family that have completed their growth cycle suggests that these species could in future establish themselves at very high latitudes.

In France, several tick species with the potential to transmit CCHFV are present more or less locally: Ixodes ricinus, Dermacentor marginatus, Dermacentor reticulatus, Haemaphysalis punctata, as well as Rhipicephalus sanguineus and Hyalomma marginatum marginatum. Recent studies have shown the presence of breeding populations of H. marginatum, notably in the French Mediterranean region.

In France, the increase in the range of these ticks could be the result of avian migrations, but also, and to a significant extent, the transport of livestock (horses and cattle). This tick was already known in Corsica, where it has been established for many decades. Given the wide distribution of its vector, the many animal species that can serve as amplifying hosts, the favorable climate and climatic conditions in several European countries bordering the Mediterranean, there is a strong possibility that the range of the CCHFV virus will expand in the future. A model study of various climatic scenarios that could occur in the habitat areas of the various ticks has shown that an increase in temperature and a decrease in precipitation in the Mediterranean region will result in a sharp increase in their establishment in these suitable habitat areas, including their expansion northwards. In Eastern Europe, the first cases have been observed in Greece, and micro-epidemics (fewer than 20 cases) have been reported sporadically in the Balkans since 2001 (Croatia, Kosovo, Macedonia). In 2010, virus-carrying H. lusitanicum ticks were first collected from a deer in the Cáceres region of Spain, revealing the presence of the virus in Western Europe. More recently, virus-carrying ticks or serology-positive animals have been identified in several regions, including the Spanish region of Huesca, close to the French border. In September 2016, two cases of human infection, including one fatality, were reported in Avila ((province of Castile and Leon, 80 km from Madrid). A suspected case was also mentioned in 2018 in Badajoz in Extremadura (southwest of Spain), and a new case was detected in June 2020 in Salamanca. A seroprevalence study carried out among blood donors showed a seropositivity rate of around 1% in this region. In France, no cases have yet been reported, but the seropositivity rate seems high in Corsica, particularly among cattle (13%), sheep and goats (2 to 3%), even if no ticks carry the virus has not yet been detected and identified. The virus present in Spain was related to strains grouped in clade III (strains from southern and western Africa). Viruses belonging to this same clade have also been identified in Morocco, in birds, which suggests a possible introduction of the virus into Spain via migratory birds. Similarly, a tick carrying a virus of this clade III was collected from a meadow cat (Saxicola rubra), a species of passerine, on the island of Ventotene, off the coast of Naples in Italy, indicating a possible presence of the virus on the Italian island. territory. The viral strains present in Corsica have not yet been identified. Avian migration routes crossing Corsica being different from those crossing Spain, it is possible that the viral strains in these regions vector, and therefore of the virus are potentially divergent. A low pathogenic strain of the virus, strain AP92, initially isolated in Vergina in Greece, was also found in Turkey and in Algeria.

What about Romania

Recent studies have shown that this zoonosis is also circulating in animals in countries such as Romania and the former Yugoslav Republic of Macedonia (Nemeth V and al. 2013, Ceianu CS and al. 2012; Mertens M and al. 2015; Bratuleanu B. and al. 2022). In the last study in Southern Romania (2022), the overall seroprevalence of CCHF in small ruminants was 37.7% (95% CI 31.7 to 43.7). This high seroprevalence to CCHFV among ruminants indicates that CCHV or a closely related virus circulates in Southern Romania.

We performed a first human seroprevalence study for CCHFV with the Danube Delta National Institute for Research and Development and Sanitary Veterinary and Food Safety, Tulcea, Romania (article submitted). Our data showed that 38% of professional exposed workers (i.e. veterinarians and animal breeders) are seropositive for CCHFV antibodies. We also demonstrate a CCHFV seroprevalence of 26% in the control group (not professionally exposed). These data suggesting that the disease could be endemic, in the South-Est Romania region and argue to be confirmed in animals.

Conclusion

The presence of the virus in western Europe and in the Balkans, suggests that more and more cases are reported in Europe so in France as well. It is therefore important to continue to deepen our knowledge of this virus and to develop new antiviral molecules, vaccine strategies and diagnostic tests.

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IN VIVO AND IN VITRO MODELS TO STUDY THE MOSQUITO-BORNE USUTU VIRUS

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Abstract

Usutu virus (USUV), a mosquito-borne zoonotic flavivirus discovered in South Africa in 1959, has spread to many European countries over the last 20 years. The virus is currently a major concern for animal health due to its expanding host range and the growing number of avian mass mortality events. Although human infections with USUV are often asymptomatic, they are occasionally accompanied by neurological complications reminiscent of those due to West Nile virus (another flavivirus closely related to USUV). The knowledge about the various study models is a helpful tools for scientific to identify the best methos for different scientific questions.

Keywords: virus, zoonotic, in vitro and in vivo models

INTRODUCTION

Usutu virus (USUV) is an arthropod-borne virus (arbovirus) belonging to the genus Flavivirus within the Flaviviridae family. As a member of the Japanese encephalitis virus (JEV) antigenic complex, USUV is closely related to numerous human and animal pathogens including West Nile virus (WNV), Murray Valley Encephalitis virus (MVEV), and St Louis encephalitis virus (SLEV). USUV is maintained in the environment through a typical enzootic cycle involving mosquitoes and birds. Since first identification in South Africa in the middle of 20th century, widespread circulation of USUV was observed in several countries. In Europe, USUV emerged in 1996 causing high numbers of bird deaths. Five years later, USUV was responsible for high mortality rate among Eurosian Blackbirds (Turdus merula) in the surrounding area of Vienna, Austria. Currently, USUV is endemic in several countries in Europe.

The clinical relevance of USUV as human pathogen has been hypothesized since the first descriptions of USUV-related infection in humans. The first report of USUV infection in humans was described in Africa at the beginning of 1980s. Thirty years later, two cases of USUV-related neuro- invasive diseases were reported in immune-compromised pa- tients in Europe (Italy). Although different human cases of USUV infection have been reported until today, the effective role of the USUV as a human pathogen has yet to be clarified.

Virus genome and structure

USUV is a small and spherical virus with a lipid envelope derived from host cell membrane. The virion is 40e60 nm in diameter and contains a positive-sense RNA genome of 11 Kb in length with no 30 poly(A) tail. Genomic organization shows a similar structure comparable to other flaviviruses. The genome consists of a single-stranded RNA genome with a 5’ cap structure, a unique open reading frame (ORF) and two untranslated regions (UTRs). The 5’ and 3’ UTRs varied respectively between 95 to 96 nt and 631 to 664 nt in length among different strains. The UTRs are involved for the translation and replication of the viral genome. The predicted ORF is translated in a unique polyprotein of 3434 amino acids that is

post-translationally processed into three structural (capsid, envelope, and pre-membrane) and eight non- structural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5). Like other mosquito-borne flavivirus, genes encoding the structural proteins are located on the 5' end of viral genome and form the virion particle. The capsid protein (C) forms the central core of the virion and is asso-ciated to the viral RNA. The envelope glicoprotein (E) mediates binding to the host cells and promotes viral entry into the host cells. The pre-membrane protein (prM) are necessary for virion assembly and maturation by assisting envelope folding. The nonstructural proteins serve to different functions during infection and their functions are deduced on the basis of the similarity with other flavivirus genomes. NS1 exists in distinct forms (i.e. cellular and secreted) and is necessary on the replication of viral genome and virion maturation. The NS2A, NS2B, NS4A and NS4B are small, hydrophobic proteins that are required for virus as- sembly and play a role in the inhibition of the IFN response. NS3 and NS5 are two proteins with different enzymatic activities: NS3 protein encodes for viral serine protease (active only with NS2B cofactor), helicase, nucleoside tri- phosphatase and RNA triphosphatase. NS5 protein encodes for a methyltransferase (MTase) at the N-terminal, while C- terminal encodes for the RNA-dependent RNA polymerase.

Life cycle, hosts, vectors

USUV was isolated for the first time from a *Culex neavei* mosquito captured near the Usutu river in Ndumu, South Africa, in 1959. Subsequently, USUV circulation in the African continent has been detected in several countries: Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Ivory Coast, Tunisia, Morocco, and Algeria. Until 2001, USUV was considered as exclusively African, non-fatal for wild birds or domestic animals, and exceptionally zoonotic. In 2001, USUV was isolated from blackbirds (*Turdus merula*) found dead during an epizootic that affected the resident passerines and *Strigiformes* in Austria. Retrospective analyses have shown that the high mortality of blackbirds in Tuscany (Italy) in 1996 was also attributed to this virus. In the following years, USUV circulation was identified in many countries in western, southern, and central Europe: United Kingdom (2001–2002), Czech Republic (2005), Hungary (2005), Poland (2006), Spain (2006) [29], Switzerland (2006), Serbia (2009–2010), Greece (2010), Germany (2011), Slovakia (2012–2014), Belgium (2012), France (2015), and The Netherlands (2016). In many of these countries, USUV has managed to establish an endemic mosquito–bird life cycle and to co-circulate with WNV.

To date, USUV has been detected in mosquitoes belonging to seven genera (*Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, and *Ochlerotatus*). However, it seems to be most often associated with *Culex pipiens*. The main natural reservoir hosts of USUV are birds; the virus presence was demonstrated to date in 101 bird species belonging to 18 orders and 38 families. However, the natural virulence spectrum of USUV seems rather limited, with a marked virulence in the European blackbird (*Turdus merula*), house sparrow (*Passer domesticus*), grey owl (*Strix nebulosa*), and common scoter (*Melanitta nigra*). In these species, prostration, disorientation, locomotor disorders, and death may occur. The two macroscopic lesions most commonly observed at autopsy are splenomegaly and hepatomegaly. Pathohistological analysis revealed inflammatory and necrotic lesions, with histiocytic and lymphoplasmacytic infiltrates, have been described in the heart, lung, liver, kidney, spleen, and brain of the infected birds. Although the virus was isolated from mammalian species, namely rodents (*Mastomys natalensis*, *Crocidura spp.*, and *Rattus rattus*) and Chiroptera (*Rousettus aegyptiacus* and *Pipistrellus pipistrellus*), no pathological signs could be observed in these hosts and their potential role as a reservoir for this arbovirus is still questionable. Other mammals, such as equids , dog, wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), tree squirrel (*Sciurus vulgaris*), Malayan tapir (*Tapirus indicus*), chimpanzee (*Pan troglodytes*), giant panda (*Ailuropoda melanoleuca*), common eland (*Taurotragus oryx*), and white rhinoceros (*Ceratotherium simum*), as well as reptiles (green lizards (*Lacerta viridis*), presented neutralizing antibodies specific for USUV and may act as incidental hosts.

In humans, USUV infection (like WNV) is usually asymptomatic. More than 80 cases of subclinical infections have been described in blood donors or healthy patients in Italy, Serbia, the Netherlands, and Germany during the surveillance of WNV circulation. Clinical disease with moderate flu-like (rash, fever, and headache) manifestations may also occur. The neurotropism of USUV represents a growing concern for human health. In more than 32 cases to date, severe neurological disorders, including facial paralysis, encephalitis, meningitis, and meningoencephalitis, in both immunocompromised and immunocompetent patients have been observed.

These severe acute human cases, along with the avian mass mortality induced by this virus in Europe and numerous similarities with WNV biology and clinical manifestations, have prompted the development of experimental models to clarify the mechanisms underlying USUV pathogenesis and transmission. Besides, given that no approved effective therapeutics and no licensed vaccines against USUV exist so far for humans or birds, some of these models were used for their development. This is the first review to focus on in vitro and in vivo models of infection with USUV and summarize their contribution to clarify USUV pathogenesis and potential countermeasures.

USUSV Cellular Tropism and In vitro models

To date, the virus has been shown to infect a large spectrum of cells from 23 mammalian species, two avian species, and one reptile (turtle, *Terrapene carolina*). The first USUV in vitro replication assay was performed in porcine kidney (PK) cells in 1969. Later, Bakonyi et al. (2005) demonstrated USUV replication in a wide range of cells. However, only African green monkey kidney cells (Vero), PK-15 pig epithelial cells, and goose embryo fibroblasts have developed cytopathic effects (CPE). Like other flaviviruses, USUV replicates efficiently in Vero and mosquito (*Aedes albopictus*) C6/36 cells, which are commonly used for virus isolation from both clinical and animal (birds/rodents/mosquito) samples and often after replication in these cells, other cellular or animal models are used. The particular susceptibility and the extent of CPE observed in Vero cells explain their use for virus culture and viral titer studies, such as 50% tissue culture infectious dose, $TCID_{50}$, and plaque reduction neutralization tests [5]. In these cells lacking the interferon (IFN)-α and IFN-β genes, USUV infection activates cellular stress and autophagy, promoting viral replication. Further, USUV can establish a persistent infection for at least 80 days and present full-length and defective viral genomes (DVGs), containing truncations at the 5′ end, which may be a key determinant in the survival and persistence of the infection. Multiple cellular systems were used primarily to investigate USUV tropism. Mammalian cells were further used to explore USUV infection neuropathogenesis, the cell-intrinsic immune response, and/or the effect of antivirals on USUV replication.

USUV shows different replication characteristics in rodent species and rodent-derived cell types. The woodchuck (*Marmota monax)* liver cells (WCH-17, ATCC No: CRL-2082), rat (*Rattus norvegicus*) brain cell line (C6), and hamster (*Mesocricetus auratus)* kidney cell line (BHK-21) were susceptible to USUV infection but did not display CPE. However, primary astrocytes, microglial cells, and neurons of a wild-type mouse (*Mus musculus*) supported efficient USUV replication and showed CPE. While a bank vole (*Myodes glareolus*) kidney cell line (BVK168, RRID: CVCL_A014) showed CEPs following USUV infection, the virus did not replicate at all in the lung cells of this animal and did not show CPE in kidney or brain cells of the common vole (*Microtus arvalis*). Likewise, USUV could infect human cells from different origins, including the upper respiratory tract, brain, and retina, but only a few of these cells exhibited CPE.

USUSV and in vivo models

Mosquito Infection Models

Before USUV emergence in Europe, only one study registered experimental infections with USUV in mosquitoes. It showed the susceptibility of *Cx. neavei* to USUV, but no effective transmission to hamsters could be demonstrated [96]. After USUV detection in dead birds and several ornithophilic mosquito species in many European countries, the vector competence of European, African, and even American mosquito populations was addressed through experimental infections of these invertebrate hosts. *Cx pipiens* has been used as the major experimental model (in 4/7 studies). This can be justified by the abundance of this vector and the fact that USUV has been frequently detected [97] and co-circulating with WNV in biotypes of this mosquito complex collected in nature. Some North American and European populations of *Cx. pipiens pipiens*, *Cx. pipiens molestus*, *Cx. quinquefasciatus*, and/or hybrid forms have shown that both European and African strains of USUV effectively infect their bodies and accumulate in their saliva under laboratory conditions. However, two UK strains of *Cx. pipiens* infected with a USUV strain of African origin showed a very low vector competence, which could be due to the genetic variability of USUV strains or mosquito populations from the same species. Further, the infectivity of USUV in *Cx. pipiens* showed a pronounced temperature dependency. A clear relationship between the virus titer in the blood sample and the infection rate of *Cx. naevi* was demonstrated. Thus, a range of factors should be carefully considered to compare the competence of a particular mosquito species for the same virus.

The vector competence of *Cx pipiens* for USUV was compared with that for WNV and

ZIKV. While none of the tested mosquitoes accumulated ZIKV in the saliva and were considered as incompetent vectors for ZIKV, *Cx. pipiens molestus* and *Cx. pipiens pipiens* were shown to be susceptible to USUV infection and to disseminate the virus in their salivary glands. The infection and transmission rates with USUV (80% and 69%, respectively) were significantly higher than with WNV (46% and 33%, respectively) under elevated temperature (28 °C) in these mosquitoes.

Two mosquito species of the genus *Aedes* were assessed for their vector competence to USUV, namely *Ae. Albopictus*, repeatedly found infected in northern Italy, and *Ae. japonicas*, which is invading Europe and disseminating USUV in Graz (Austria). North American and European populations of *Ae. albopictus* appeared to be experimentally incompetent vectors for USUV and the detection of USUV from field-collected *Ae. albopictus* was explained by simple recent engorgement from viremic birds. In contrast, field-collected *Ae. japonicus* mosquitoes from the Netherlands showed USUV-positive saliva after 14 days at 28 °C, and, therefore, could play a role in the transmission cycle of the virus in Europe.

Bird Infection Models

USUV is highly pathogenic in some wild and captive bird species, due to its extensive tropism and virulence in various tissues and organs. Thus, these hosts are the most plausible in vivo models to characterize the pathogenesis of USUV infection. Besides, USUV has very selective pathogenicity within these hosts, including members from the same bird family. For instance, the natural USUV infection might be unapparent in domestic geese (*Anser anser f domestica*), while in another anatid, the common scoter (*Melanitta nigra*), USUV could result in fatal infection. Thus, it would be tempting to use such models to identify molecular determinants associated with virulence and host tropism, which may help anticipate key events leading to the possible emergence of USUV in new hosts and territories. However, to date, only three avian species have been used to address the susceptibility of these hosts to USUV infection. Domestic chicken (*Gallus gallus domesticus*) and geese (*Anser anser f domestica)* were reported to resist USUV infection under experimental conditions. More recently, the domestic canary (*Serinus canaria*), a passerine species, such as highly susceptible blackbirds, showed a mortality rate of 30% after infection via the intraperitoneal (IP) route with two different doses (10^3 and 10^6 $TCID_{50}$) of a European strain of USUV. In addition, USUV induced a specific humoral immune response in almost all the survivors after 15 days of infection. Chicken and goose embryos were also tested for their susceptibility to the virus. While USUV showed viral replication in goose embryos tissues, some studies showed that chicken embryos were resistant to infection, while one recent paper demonstrated that they are highly susceptible to USUV infection in a dose-dependent manner. These contradictory results could be explained by the genetic variability of the USUV strains and the differences in the genetic backbone of the eggs used, conditioning the immune response between breeds/individuals of the same bird species.

In addition to their susceptibility to USUV, the avian models available to date to study USUV, namely chicken and goose embryos and domestic canaries, have shed new light on USUV pathogenesis and transmission in birds. Similar to WNV, death due to USUV in domestic canaries was more likely attributed to a multi-systemic failure than to a pure neurologic disease, and the virus infected all major systems and a wide variety of cell types. The myocardial cells strongly supported viral replication, as viral antigens were systematically detected by immunohistochemistry (IHC) in the experimentally infected chicken embryos and canaries.

In all these three models, USUV displayed a particular tropism for the eyes. Visual impairment and ocular lesions have been described following infection of birds with other flaviviruses, such as WNV. A vision assessment should be performed during future experimental infections in vivo with USUV.

Immunocompetent Models

Developing an animal model relevant to human USUV infection seems to be extremely challenging because experimental infections have shown that immunocompetent mammals rarely develop severe forms of USUV disease. African fruit bats (*Eidolon helvum*) and (*Rousettus aegyptiacus*) and the Angolan free-tailed bat (*Tadarida (Mops) condylura*) were not susceptible at all to USUV injected intraperitoneally. Guinea pigs showed only an antibody response following intracerebral inoculation with the USUV SAAR-1776 strain. The Abyssinian grass rat (*Arvicanthis abyssinicus*) could exhibit a trace of viremia 1–2 days after IP inoculation of USUV (unknown strain) and developed neutralizing antibodies. Immunocompetent mouse models showed different susceptibilities to USUV infection across the studies. Intracerebral (IC) inoculation of USUV

successfully induced signs and mortalities in neonatal and 3–4 weeks-old immunocompetent mice. However, this injection route is not pertinent enough to describe USUV neuropathogenicity, as it only models viral neurovirulence. Thus, peripheral inoculation (e.g., subcutaneous SC or IP) was more commonly used to reflect both USUV neurovirulence and neuroinvasiveness. Experimentally, no mortality was observed following IP infection with USUV of Naval Medical Research Institute (NMRI) mice aged over 2 weeks with a European USUV strain. Similarly, the USUV prototype strain SAAR-1776 showed no pathogenicity in adult Swiss mice via the IP route. However, in the study of Diagne et al. , both SC and IP infections of this strain resulted in a 30% and 50% mortality, respectively, in 3–4-week-old Swiss Webster (CFW) mice after 15 days of infection. Likewise, in the same study, the IP inoculation of a mouse-derived USUV strain induced a 10% mortality 10 days after infection. USUV infection failed to elicit pathogenicity in wild-type 129/Sv mice via the IP and IN routes but induced a typical neurological disease in a single 129/Sv mouse infected via the ID route. These findings indicate that the outcome of USUV infection in immunocompetent mice depends on several factors, such as the strain of virus or mouse used. Age is also a key determinant of susceptibility to USUV and suckling mice are generally much more susceptible than older animals. NMRI suckling mice showed 100% mortality with as few as 10^3 Plaque-forming units (PFU) after 11 days of infection. Dose-dependent mortality was observed in Swiss suckling mice, as 84% and 40% survived the infection with 10^2 and 10^4 PFU, respectively. The higher predisposition of newborn neurons to apoptosis and the incomplete development of the BBB are plausible explanations for this difference in the infection outcome. Although immunocompetent models present limitations regarding their efficiency to manifest the USUV-associated disease, they are important to obtain knowledge about USUV pathogenesis under functional innate and adaptive immune responses of the host. In immunocompetent mice, USUV infection induced clinical signs, such as disorientation, depression, paraplegia, and paralysis, associated with extensive neuronal death, including both necrosis and apoptosis in the brain. Alternatively, no trace of viral infection or a simple detection of the USUV genome in brain portions of USUV-infected mice were described after 15 days post-infection, without the induction of specific clinical signs. These models reflect the infection in humans, in which most individuals show subclinical infections but rare cases can develop clinical disease.

CONCLUSIONS

USUV it can be currently considered as a leading model for the study of flaviviral pathogenesis and the development of prophylactic and therapeutic solutions against these more pathogenic flaviviruses. Indeed, it can be handled under level 2 biosafety conditions; besides, field strains are easily accessible and have a certain degree of natural genetic variation. Despite these advantages, little effort has been made so far to the development of in vitro and in vivo models for the study of this neurotropic virus, given that human infections most often remain asymptomatic, or with a benign clinical expression and only a few bird species naturally develop severe forms of USUV virus disease.

The different in vitro and in vivo models are essential to investigate the specific pathogenicity, virus transmission routes, and host tropism.

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ACUTE KIDNEY INJURY IN A DOG DIAGNOSED WITH LEISHMANIASIS: CASE REPORT

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Abstract

A 3 years old, 40.8 kg, intact male American Bully, diagnosed with acute kidney injury in a private clinic in Serbia was referred for hemodialysis therapy. The dog was presented with the following symptoms: lethargy, appetite loss, vomiting, diarrhea, weight loss, dehydration (8-10%, Considerable loss of skin turgor), rectal temperature of 38.8ºC and dry mucous membranes. Arterial blood pressure was elevated 218-220 mmHg systolic, using Doppler method. The biochemistry revealed elevated ALT 191 (RR: 10-118 U/L), AMY 1390 (RR: 200-1200 U/L), BUN 94 (RR: 7-25 mg/dL), CREA 3.6 (RR: 0.4-1.2 mg/dL), PHOS 9.8 (RR: 2.9-6.6 mg/dL), GLU 121 (RR: 60-110 mg/dL), K 3.3 (RR: 3.4-5.6 mmol/L). Urine analysis was performed with UPC 0.2-0.5 (borderline proteinuric), pH 5.5, microalbumin >25 mg/L, creatinine >26.4 mmol/L. The infestation with Leishmania infantum was confirmed using quantitative PCR. The patient was stabilized using fluid therapy and parenteral feeding. Hemodialysis was decided as an extracorporeal replacement therapy for sustaining renal function. A central venous catheter was placed under a light sedation with oxygen therapy. Hemodialysis was performed for three times in a period of 11 days.

A key treatment for acute kidney injury in canine patients is represented by intensive care, fluid therapy and hemodialysis therefore, the values were reduced in BUN from 94 mg/dL to 30 mg/dL and CRE from 3.6 mg/dL to 1.8 mg/dL. The BUN and CREA reached normal values 39 days after that the patient was discharged.

Key words: hemodialysis, canine, Leishmania, BUN, CREA

Leishmania is a genus of parasitic protozoa (family *Trypanosomatidae*) that has significant attention in the field of infectious diseases, particularly due to its complex life cycle and diverse clinical manifestations. This intracellular parasite is responsible for causing a group of diseases konwn as leishmaniasis. *Leishmania infantum* has a biphasic life cycle, alternating between two main forms – visceral and cutaneous.

Transmission to mammalian hosts primarily occur through the bite of infected sandflies, which convey the promastigotes into the host's bloodstream. These promastigotes are subsequently phagocytosed by macrophages, converting into the amastigote form and establishing infection. [Gharbi M. *et al*, 2015]

Visceral leishmaniasis caused by *Leishmania infantum* is characterized by a wide spectrum of symptoms, often manifesting as prolonged fever, splenomegaly, hepatomegaly, anemia, glomerulonephritis, interstitial nephritis, amyloidosis, weight loss and compromised immune function.

Leishmaniasis is a serious medical condition that affects the body through acute complications to the renal and hepatic function. Left untreated, leishmaniasis can be fatal, making early diagnosis and treatment, crucial for the patient with acute kidney injury. [Mann S. *et al*, 2021]

Hemodialysis is a therapeutic procedure that uses the extracorporeal circulation of a patient's blood to improve azotemia, fluid overload, electrolyte and acid-base abnormalities characteristic of the uremic syndrome. Hemodialysis is used for the management of acute and chronic renal injury that is refractory to

conventional medical therapy. Additional applications include acute intoxications (e.g. ethylene glycol poisoning) and preoperative conditioning of renal transplant recipients. [Ștefănescu, A. *el al*, 2017, 2018]

Hemodialysis is a special procedure that requires an extensive array of sophisticated delivery equipment and specifically trained and dedicated staff to perform, monitor and ensure the integrity and safety of the procedure in critically ill patients. [Elliott D.A., 2000]

For the moment, there are two types of hemodialysis: intermittent and continuous hemodialysis. Intermittent hemodialysis (IHD) is a renal replacement therapy that is defined by short and efficient hemodialysis sessions with the goal of removing endogenous or exogenous toxins from the bloodstream. IHD is indicated in cases of acute azotemia, electrolyte abnormalities or acidosis unresponsive to medical management. When patients undergo IHD, their blood is removed from their bodies and run through an extracorporeal circuit. The slow and gradual nature of the technique provides better control of electrolytes and acid-base balance. [Bellomo R. *et al*, 1995]

The goal of IHD is to make big changes in a patient's uremia, acid-base and fluid status over short periods using diffusion.

The continuous operation more closely approximates the functioning of a normal kidney. [Clark W.R. *et al*, 1994]. Continuous renal replacement therapy (CRRT) is a continuous process and once it begins, therapy continues until renal function is restored or the patient is transitioned to intermittent dialysis. The most common indication for CRRT is the treatment of acute kidney injury (AKI) in which renal function is expected to return in the near future or for patients who are to be transitioned to IHD.

Vascular access is the first and the most basic requirement of successful extracorporeal renal replacement therapy (ERRT). An adequately functioning dialysis catheter allows for smooth and efficient patient management. Various materials can be used to make a catheter that is minimally thrombogenic, flexible, and non-irritating to the vessel wall. [Chalhoub S. *et al*, 2011]

IHD is designed as a more efficient modality than continuous renal replacement therapy (CRRT), meaning that IHD sessions remove small dialyzable molecules (blood urea nitrogen [BUN], creatinine, phosphorus, electrolytes, and certain drugs and toxins) from the bloodstream more rapidly than CRRT. [Bloom C.A. *et al*, 2011]

MATERIAL AND METHOD

A 3 years old, 40.8 Kg, intact male American Bully, diagnosed with acute kidney injury was referred to a private clinic for hemodialysis therapy on June the 4th, 2023. The dog was presented with the following symptoms: lethargy, appetite loss, vomiting, diarrhea, weight loss, dehydration (8-10%, considerable loss of skin turgor), rectal temperature of 38.8^{o}C and dry mucous membranes. Results from a complete blood cell count (CBC), biochemistry, and urine analysis submitted at that time were abnormal.

Abdominal ultrasound showed mild modification in kidneys, a regular shape, altered corticomedullary ratio, slightly hyperechogenic appearance, renal pyramids highlighted with no microlithiasis or dilatation of the renal pelvis. The immunofluorescence antibody test (IFAT) was performed for detection of anti-Leishmania antibodies (*figure1*). [Proverbio D. *et al*, 2014]

Hemodialysis, hydro-electrolytic rebalancing and partial parenteral nutrition were the main goals of the complex therapy. Rehydration was established by fluid therapy with Ringer continous rate of infusion (CRI), (rate and dosage: 7 ml/kg/h) for rebalancing and partial parenteral micronutrition based on levo-microamino acids (rate and dosage: 6 ml/kg/24 h). Enteric dialysis supplements, calcium based phosphorus binders, renal diet and nutritional supplements were introduced as adjuvants in the therapy for supporting kidney functions. Telmisartan was used in the treatment of hypertension.

After 24 hours biochemistry showed the following results: BUN 88 mg/dL, CREA 3.6 mg/dL and PHOS 3.6 md/dL.

Hemodialysis was decided in order to improve and support renal function. A central venous *Joline High Flow Double Lumen ST 13 Fr* catheter was placed under a light sedation with alfaxalone (dosage: 3mg/kg) which was administered intramuscularly and butorphanol (dosage: 0.3 mg/kg) administered intravenously. Intermittent hemodialysis was performed with an A/V set and a high flow dialyzer with a surface of 1.5 m^2. The volume of the circuit was 232 ml. Urea reduction ratio was calculated for 50% and the duration of therapy was 6 hours.

After the first hemodialysis session the pacient had BUN 43 mg/dL, CREA 3.2 mg/dL and phosphorus 7.2 mg/dL. After 9 days, with 3 hemodialysis sessions, blood biochemistry showed the followings: BUN 30 mg/dL, CREA 1.8 mg/dL and Phosphorus 6.4 mg/dL. Hemodialysis therapy was withholded for 72 hours between the 2nd and the 3rd session. During hospitalization, the adjuvant therapy was never suspended and was prolonged and adjusted in correlation with the subsequent analyses. BUN (9.8 mg/dL), CREA (0.9 mg/dL) and phosphorus (4.6 mg/dL) reached normal values

within 39 days after that the patient was discharged.

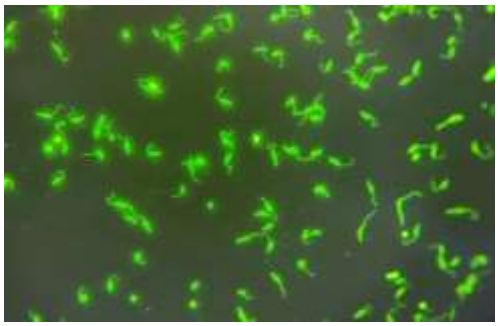

Figure 1. Immunofluorescence antibody test (IFAT), on optic-microscopy, technique for detection of anti-Leishmania antibodies. (by courtesy of Exavet)

RESULTS AND DISCUSSIONS

On 4th of June 2023, the patient had elevated blood biochemistry and grade 3 acute kidney injury CREA 3.6 (RR: 0.4-1.2 mg/dl), BUN 94 (RR: 7-25 mg/dl), phosphorus 9.8 (RR: 2.9-6.6 mg/dl), mild hypokalemia K^+ 3.3 (RR: 3.4-5.6 mmol/L) and elevated liver transaminase ALT 191 (RR: 10-118 U/L). Mild elevated GLU 121 (RR: 60-110 mg/dL). The AMYL was 1390 (RR: 200-1200 U/L), Total Protein 8.6 (RR: 5.4-8.2 mg/dL), Globuline 5.8 (RR: 2.3-5.2 mg/dL). Results from complete blood cell count (CBC) showed MCHC 29.7 (RR: 31-39 g/dl) and RDWc 20.3 (RR: 14-20%). Urine analysis was carried out from urine obtained through echo-guided cystocentesis and showed UPC ratio of 0.2-0.5 (borderline proteinuric), pH 5.5, microalbumin >25 mg/L, creatinine >26.4 mmol/L. Abdominal ultrasound revealed a small amount of free fluid in the hepato-diaphragmatic quadrant, moderate modification in kidneys, hepatomegaly, splenomegal and mild inflammation of small intestines. The patient was presented with the following symptoms: lethargy, appetite loss, vomiting, diarrhea, weight loss, dehydration (8-10%, considerable loss of skin turgor), rectal temperature of 38.8^{o}C and dry mucous membranes. Arterial blood pressure was elevated 218-220 mmHg systolic, using Doppler method.

In the first day, 24 hours prior intermittent hemodialysis (IHD) session, the patient was submitted to intravenous fluidotherapy for electrolyte rebalancing and partial parenteral micronutrition based on levo-microamino acids. Calcium based phosphorus binders, renal diet and nutritional supplements were introduced as adjuvants in the therapy in order to support kindey functions. Telmisartan was used in the treatment for hypertension and border-proteinuria.

After 24 hours of therapy, the blood biochemistry was: CREA 3.6 (RR: 0.4-1.2 mg/dl), BUN decreased to 88 (RR: 7-25 mg/dl), phosphorus slightly increased to 10.1 (RR: 2.9-6.6 mg/dl), K^+ increased to a normal value of 3.5 (RR: 3.4-5.6 mmol/L), elevated liver transaminase ALT decreased to 178 (RR: 10-118 U/L) and GLU increased to 125 (RR: 60-110 mg/dL).

Hemodialysis was decided as an extracorporeal renal replacement therapy. After 24 hours of fluidtherapy, a central venous catheter was placed under a light sedation and supplimented with oxygen, using aseptic technique (the use of surgical scrub and sterile surgical technique during catheter placement, as well as the use of sterile gloves, surgical scrub, and the careful handling of catheter line during the procedure).

After the first IHD session, on 5th of June the blood tests were: CREA decreased to 3.2 (RR: 0.4-1.2 mg/dl), BUN decreased to 43 (RR: 7-25 mg/dl), phosphorus decreased to 7.2 (RR: 2.9-6.6 mg/dl), K^+ increased to a normal value of 4.4 (RR: 3.4-5.6 mmol/L), ALT decreased to 163 (RR: 10-118 U/L) and GLU decreased to a normal value of 101 (RR: 60-110 mg/dL). During the IHD, the patient was stable with no notable clinical event and he received continuous sustaining therapy until the next IHD session. The arterial blood pressure was still high - mildly decreased: 170-180 mmHg systolic, using Doppler method.

After the second session of IHD on 6th of June, blood work were: CREA decreased to 3.1 (RR: 0.4-1.2 mg/dl), BUN decreased to 35 (RR: 7-25 mg/dl), phosphorus increased to 8.0 (RR: 2.9-6.6 mg/dl), K^+ slightly decreased still to a normal value of 4.0 (RR: 3.4-5.6 mmol/L), ALT decreased to 141 (RR: 10-118 U/L) and GLU remain to a normal value of 106 (RR: 60-110 mg/dL).

It was decided to pause hemodialysis for 72 hours and give the patient an intensive and specific intravenous therapy with fluids for electrolyte rebalancing and partial parenteral micronutrition based on levo-microamino acids. Calcium based phosphorus binders and nutritional supplements were given as adjuvants in the therapy for supporting kidney functions.

Blood biochemistry through this 72 pause were: on 7th of June - CREA increased to 3.4 (RR: 0.4-1.2 mg/dl), BUN increased to 37 (RR: 7-25 mg/dl), phosphorus remain the at the same value of 8.0 (RR: 2.9-6.6 mg/dl), K^+ increased but still to a normal value of 3.8 (RR: 3.4-5.6 mmol/L), ALT decreased to a value of 132 (RR: 10-118 U/L) and GLU remains to a normal value of 109 (RR: 60-110 mg/dL). The complete blood count (CBC) showed: mild decrease in RBC to 5.43 (RR: 5.5-8.5 10^12/L), a decrease in HGB to 14.36 (RR: 12-

18 g/L) and PLT decreased to 54 (RR: 150-500 10^9/L). A recheck of abdominal ultrasound was taken and the morphological changes found in the first ultrasound check, were still noticed, but without free fluid in the abdominal cavity and the inflammatory reaction of intestines was mildly decreased. On 8th of June - CREA increased to 3.6 (RR: 0.4-1.2 mg/dl), BUN increased to 39 (RR: 7-25 mg/dl), phosphorus decreased to 7.4 (RR: 2.9-6.6 mg/dl), K^+ slightly decreased still to a normal value of 3.5 (RR: 3.4-5.6 mmol/L), ALT decreased to a normal value of 108 (RR: 10-118 U/L) and GLU remain into the normal range reference. On 9th of June - CREA decreased to 3.0 (RR: 0.4-1.2 mg/dl), BUN decreased to 38 (RR: 7-25 mg/dl), Phosphorus increased to 7.7 (RR: 2.9-6.6 mg/dl), K^+ slightly decreased to 3.2 (RR: 3.4-5.6 mmol/L), ALT and GLU remain into the normal range reference. During 72 hours of intravenous intensive therapy and oral medication, the patient was stabile, normothermic, with good urinary output, arterial blood pressure with values within 150-160mmHg, systolic, using Doppler method, with the appetite partialy recovered and good general status.

It was decided to take the 3rd session of hemodialysis on 10th of June and the blood parameters were decreased as follows: CREA 2.7 (RR: 0.4-1.2 mg/dl), BUN 34 (RR: 7-25 mg/dl), phosphorus 7.6 (RR: 2.9-6.6 mg/dl), K+ 2.8 (RR: 3.4-5.6 mmol/L), ALT and GLU remain into the normal range reference.

The patient's general condition has improved significantly and continued with the fluid therapy and partial parenteral nutrition among the adjuvant therapy for supporting kidney functions until the patient was discharged.

On the 13th of June 2023, when the patient was discharged the blood biochemistry were: CREA decreased to 1.8 (RR: 0.4-1.2 mg/dl), BUN decreased to 30 (RR: 7-25 mg/dl), phosphorus decreased to a normal value of 6.4 (RR: 2.9-6.6 mg/dl), K^+ decreased to 3.3 (RR: 3.4-5.6 mmol/L), ALT increased to a value of 139 (RR: 10-118 U/L) and GLU remains to a normal value. The CBC showed mild decrease in RBC to 5.23 (RR: 5.5-8.5 10^12/L), a decrease in HGB to 10.3 (RR: 12-18 g/L) and HCT 35.24% (RR: 37-55%); MCHC 29.3 (RR: 31-39 g/dl) and NEUT 14.83 (RR: 3-12 10^9/L) with WBC 18.57 (RR: 6-1710^9/L).

The decrease in BUN, CREA and phosphorus were quite remarkable, the BUN decreasing from 94 mg/dL to 30 mg/dl, CREA from 3.6 mg/dl to 1.8 mg/dl and phosphorus from 9.8 mg/dl to 6.4 mg/dl in 3 sessions of hemodialysis and adjuvant therapy in 9 days, the time that patient was hospitalized to the clinic (*figure 2*, *figure 3*, *figure 3*).

After 39 days since the patient was discharged with all the recommendation for a sustained therapy at home, it was observed a decrease to a normal value of CREA to 0.9 (RR: 0.4-1.2 mg/dl), BUN to 9.8 (RR: 7-25 mg/dl) and Phosphorus to 4.6 (RR: 2.9-6.6 mg/dl).

Canine leishmaniasis is expanding to countries where it was previously unknown due to a number of factors, such as climate change and the import of dogs from endemic areas. Leishmaniasis is a serious condition that generates kidney and liver impairment. Kidney involvement appears in infected dogs with glomerulonephritis which is associated with immune complexes and can progress to acute kidney injury. Renal injury is the main death cause in canine leishmaniasis.

The sooner the patient is submitted to hemodialysis therapy, the sooner it will recover. Hemodialysis also has the benefit of correcting other metabolic disturbances and supporting the kidney function during recovery.

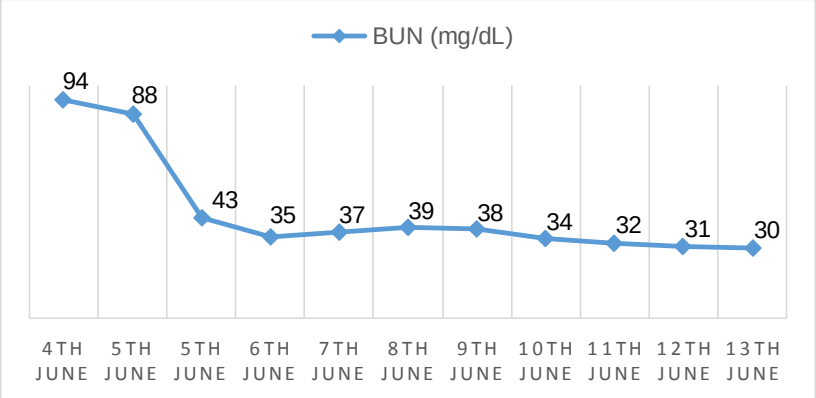

Figure 2. Evolution of BUN during hospitalization period. (original)

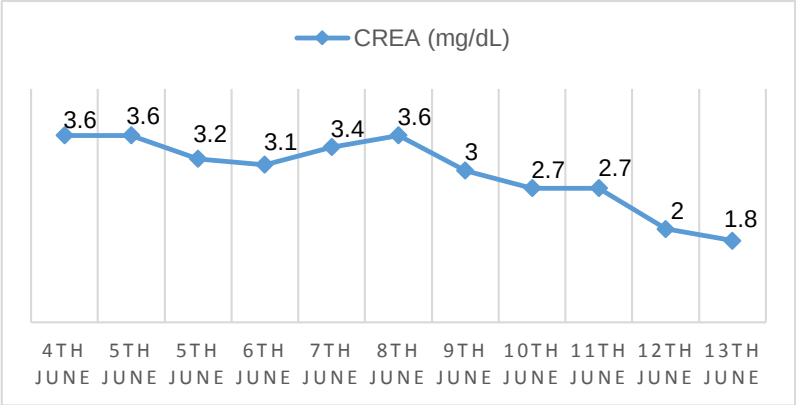

Figure 3. Evolution of CREA during hospitalization period.(original)

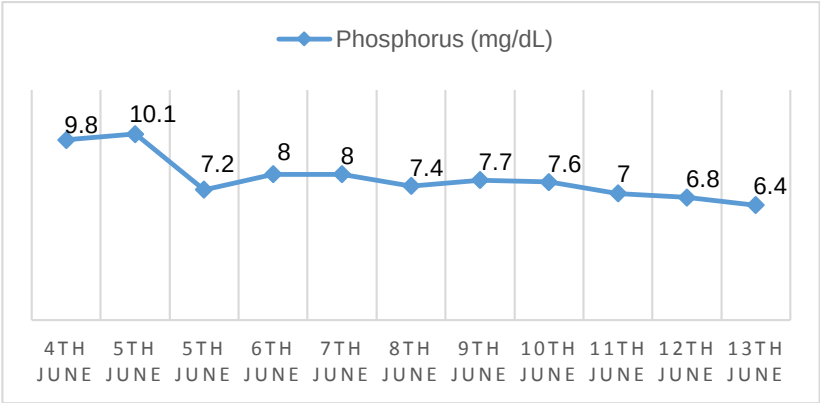

Figure 3. Evolution of Phosphorus during hospitalization period.(original)

CONCLUSIONS

In critical ill patients (especially in patients diagnosed with Leishmaniasis and acute renal injury) intensive care therapy should be initiated as soon as possible and urea and phosphorus levels should be decreased quickly.

Hemodialysis is a highly effective treatment in patients with acute kidney injury, being able to remove the toxin and certain metabolites from the blood. IHD is a useful and feasible modality to improve outcome in dogs with acute kidney injury that do not respond adequately to medical management. The decision to pursue hemodialysis in patients with acute or acute on-chronic kidney injury should be made as quickly as possible to improve the likelihood of a successful outcome. IHD requires thorough understanding of renal physiology, as well as the principles and machinery involved in dialysis. If it is used properly, hemodialysis is a life-saving procedure.

Elevated levels of creatinine and urea, hyper-/hypokalemia, hyperphosphatemia, or metabolic acidosis can be solved using hemodialysis and adjuvant treatment. Also, it has a good therapeutic effect in acute liver failure, cleaning the high levels of transaminase.

In conclusion, based on all data presented above, the process of decreasing BUN, CREA, ALT and phosphorus consisted in 48 days and 3 sessions of hemodialysis from the first day of hospitalization in the clinic, until the parameters were within range references.

For this case, a key treatment for acute kidney injury was represented by intensive care, fluid therapy and hemodialysis which stands for a good prognosis and maintaining a positive evolution for this patient.

Patients should be discharged when all intravenous support was tapered out and they are stable under oral treatments. Follow-up blood, urine and serology tests are mandatory in the next 6 months after hospital discharge.

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RESEARCHES REGARDING THE PARACLINICAL CORRELATIONS BETWEEN URINALYSIS AND WATER INTAKE IN CATS WITH FELINE UROLOGIC SYNDROME

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Abstract

The research aimed the direct correlations between the water composition and feline urological syndrome prognosis. The study was conducted on 60 patients aged between 1 and 10 years divided into two batches of 30 each. The first batch (LOT1) was represented by 30 oliguric patients, with a value of urinary specific gravity (USG) >1.055, an acidic pH and a urinary protein/creatinine ratio (UPC) >0.2, and the second batch (LOT2) was represented by 30 polyuric patients, with a urinary specific gravity (USG) <1.035, a neutral or alkaline pH and a urinary protein/creatinine ratio (UPC) <0.2 or borderline (0.2-0.4). In the first batch (LOT1), alkaline water without sodium and potassium, was administered. The water was based on a salt-free formula with a pH of 8.0 and 10 ppm of potassium. In the second batch (LOT2), a neutral water with potassium was used. The water was based on a formula with salts (magnesium citrate, calcium acetate and sodium bicarbonate), with a pH of 7.0 and 12.5 ppm potassium. In both batches, the evaluation of the USG, the pH and the UPC was carried out for 180 days, at 30, 90 and 180 days. Hydration is an essential component in the management of patients diagnosed with feline urological syndrome. Administering an alkaline hydric diet in patients with aciduria is a solution to counteract the effect of metabolism on urinary pH. Potassium supplementation in polyuric patients is a beneficial solution in hypokalemia therapy. Potassium restriction in oliguric patients is a beneficial solution in the therapy of hyperkalemia.

Key words: urinalysis, water, feline, urological, syndrome

Hydration is defined by the amount of water in the body, and maintaining normal hydration is essential for felines. A variety of vital body functions are closely related to adequate water intake, such as regulating body temperature, maintaining normal electrolyte concentrations, digesting food, and delivering oxygen and other nutrients to organs. [Zanghi B., 2017]

Increasing water intake in cats is a primary management strategy for various conditions. Patients are prone to dehydration from polyuria, hyperthyroidism, chronic kidney disease, and diabetes insipidus. Patients who develop feline lower urinary tract are also prone to dehydration. These include all conditions of the urinary bladder, urethra, such as bacterial infections, urethral plugs, urolithiasis, idiopathic cystitis, bladder neoplasia. [Buckley C.M. et al., 2011]

Numerous strategies are described in the literature to increase fluid intake in cats. These include increasing the amount of wet food in the diet, providing fresh water at all times, and providing supplemental water through various methods such as dynamic water fountains. [Robbins M.T., 2019]

Water is distributed in different compartments in the body. About 2/3 of the total water in the body belongs to the intracellular fluid compartment. This fluid is inside the cells in the body. The other third belongs to the extracellular fluid compartment, which includes interstitial fluid and plasma. About 2% of the total water is the transcellular fluid compartment, and it consists of cerebrospinal, gastrointestinal, respiratory and synovial fluid. Fluids and electrolytes can move from one compartment to another to support homeostasis. [Wellman M.L. et al., 2006]

There are no studies that conclusively demonstrate how much water cats need on a daily basis. Most sources available in the literature agree that an adult cat needs approximately 44 to 66 ml/kg/day. Kittens need a relatively larger amount of water and a dose of 66-88 ml/kg/day is recommended. These figures represent the total

water requirement, including drinking water, food water and metabolic water. [Rossi T.A., Ross, 2008; Pachel C., Neilson J., 2010]

The increased incidence of cases with feline urological syndrome (FUS), the conditions that generate this pathology, the inadequate water intake and the improvement of therapeutic protocols, were the basis for the initiation of these researches. Patients should also have a complete urinalysis performed at regular intervals. The research of this paper has the primary goal of saving the lives of companion animals by perfecting hydration methods and represents a starting point for the appropriate management of patients diagnosed with feline urological syndrome.

MATERIAL AND METHOD

This study was conducted on a number of 60 feline patients aged between 1 and 10 years. The 60 patients were divided into two groups of 30 each.

The first batch (LOT1) was represented by 30 oliguric patients, with a value of urinary specific gravity (USG) >1.055, an acidic pH and a urinary protein/creatinine ratio (UPC) >0.2. The patients were aged between 1 and 10 years, of which, 23 males and 7 females.

The second batch (LOT2) was represented by 30 polyuric patients, with a urinary specific gravity (USG) <1.035, a neutral or alkaline pH and a urinary protein/creatinine ratio (UPC) <0.2 or borderline (0.2-0.4). The age of the patients in this batch was between 1 year and 10 years, of which 24 were male and 6 were female.

In the first batch (LOT1), alkaline water without sodium and potassium, was administered. The water was based on a salt-free formula with a pH of 8.0 and 10 ppm of potassium.

In the second batch (LOT2), a neutral water with potassium was used. The water was based on a formula with salts (magnesium citrate, calcium acetate and sodium bicarbonate), with a pH of 7.0 and 12.5 ppm potassium.

In both batches, the evaluation of the USG, urinary pH and the UPC was carried out for 180 days, at 30, 90 and 180 days.

All urine samples were collected sterilely by cystocentesis, under ultrasound guidance.

The collected urine sample was instilled on a urine strip, then processed by an automatic urine biochemistry machine.

The result of the urine tests was provided within 60 seconds.

The urine strips used have the ability to measure 14 urinary biochemical parameters (e.g.: leukocytes, ketone bodies, urobilinogen, glucose, pH, urine density, etc.).

At the same time, this device also calculates the protein/creatinine ratio (UPC).

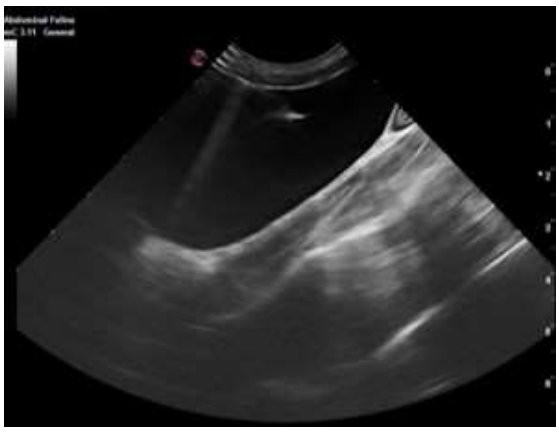

Figure 1. Ultrasound guided cystocentesis (orig.)

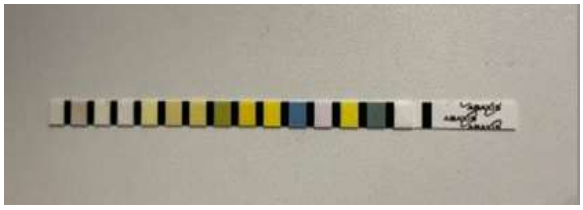

Figure 2. Urine strip with the 14 parameters measured. (orig.)

Figure 3. Automatic urine biochemistry machine. (orig.)

Patients in both batches (LOT 1, n=30; LOT 2, n=30) were administered daily 44-66 ml/kg of water.

RESULTS AND DISCUSSIONS

At presentation, all patients from batch LOT 1 (n=30), had an USG between 1.060 (n=14) and 1.055 (n=16). (Figure 4)

On day 30, patients from batch LOT 1 (n=30) had USG between 1.040 and 1.055, as follows: 1.040, n=1; 1.045, n=3; 1.050, n=18; 1.055, n=8. (Figure 4)

On day 60, patients from batch LOT 1 (n=30) had USG between 1.040 and 1.050, as follows: 1.040, n=12; 1.045, n=17; 1.050, n=1. Figure 4

On day 90, patients from batch LOT 1 (n=30) had USG between 1.035 and 1.045, as

follows: 1.035, n=11; 1.040, n=15; 1.045, n=4. (Figure 4)

On day 180, patients from batch LOT 1 (n=30) had USG between 1.035 and 1.045, as follows: 1.035, n=15; 1.040, n=15. (Figure 4)

At presentation, and also on day 30 all patients from batch LOT 1 (n=30), had an UPC ratio >0.2. (Table 1)

On day 60, patients from batch LOT 1 (n=30) had an UPC ratio as follows: >0.2, n=18; <0.2, n=12. (Table 1)

On day 90 and also on day 180, all patients from batch LOT 1 (n=30), had an UPC ratio <0.2. (Table 1)

At presentation, all patients from batch LOT 1 (n=30), had an urinary pH between 5.0 and 6.5 as follows: pH 5.0, n=5; pH 5.5, n=19; pH 6.0, n=4; pH 6.5, n=2. (Figure 5)

On day 30, patients from batch LOT 1 (n=30) had an urinary pH between 5.0 and 6.5, as follows: pH 5.0, n=14; pH 5.5, n=14; pH 6.0, n=0; pH 6.5, n=2. (Figure 5)

On day 60, patients from batch LOT 1 (n=30) had an urinary pH between 5.5 and 6.5, as follows: pH 5.5, n=21; pH 6.0, n=7; pH 6.5, n=2. (Figure 5)

On day 90, patients from batch LOT 1 (n=30) had an urinary pH between 6.0 and 6.5, as follows: pH 6.0, n=26; pH 6.5, n=4. (Figure 5)

On day 180, patients from batch LOT 1 (n=30) had an urinary pH between 6.0 and 6.5, as follows: pH 6.0, n=26; pH 6.5, n=4. (Figure 5)

At presentation, patients from batch LOT 2 (n=30) had USG between 1.010 and 1.025, as follows: 1.010, n=8; 1.015, n=9; 1.020, n=10; 1.025, n=3. (Figure 6)

On day 30, patients from batch LOT 2 (n=30) had USG between 1.010 and 1.030, as follows: 1.010, n=4; 1.015, n=8; 1.020, n=8; 1.025, n=8; 1.030, n=2. (Figure 6)

On day 60, patients from batch LOT 2 (n=30) had USG between 1.015 and 1.035, as follows: 1.015, n=2; 1.020, n=6; 1.025, n=12; 1.030, n=4; 1.035, n=6. (Figure 6)

On day 90, patients from batch LOT 2 (n=30) had USG between 1.025 and 1.040, as follows: 1.025, n=2; 1.030, n=2; 1.035, n=18; 1.040, n=8. (Figure 6)

On day 180, patients from batch LOT 2 (n=30) had USG between 1.035 and 1.040, as follows: 1.035, n=18; 1.040, n=12. (Figure 6)

At presentation, and also on day 30 all patients from batch LOT 2 (n=30), had an UPC ratio between 0.2 and 0.4. (Table 2)

On days 60, 90 and 180, all patients from batch LOT 2 (n=30), had an UPC ratio <0.2. (Table 2)

At presentation, all patients from batch LOT 2 (n=30), had an urinary pH between 7.0 and 8.0 as follows: pH 7.0, n=11; pH 7.5, n=15; pH 8.0, n=4. (Figure 7)

On day 30, patients from batch LOT 2 (n=30) had an urinary pH between 6.0 and 7.5, as follows: pH 6.0, n=1; pH 6.5, n=8; pH 7.0, n=13; pH 7.5, n=8. (Figure 7)

On day 60, patients from batch LOT 2 (n=30) had an urinary pH between 6.0 and 7.0, as follows: pH 6.0, n=1; pH 6.5, n=20; pH 7.0, n=9. (Figure 7)

On day 90, patients from batch LOT 2 (n=30) had an urinary pH between 6.0 and 7.0, as follows: pH 6.0, n=14; pH 6.5, n=13; pH 7.0, n=3. (Figure 7)

On day 180, patients from batch LOT 2 (n=30) had an urinary pH between 6.0 and 6.5, as follows: pH 6.0, n=20; pH 6.5, n=10. (Figure 7)

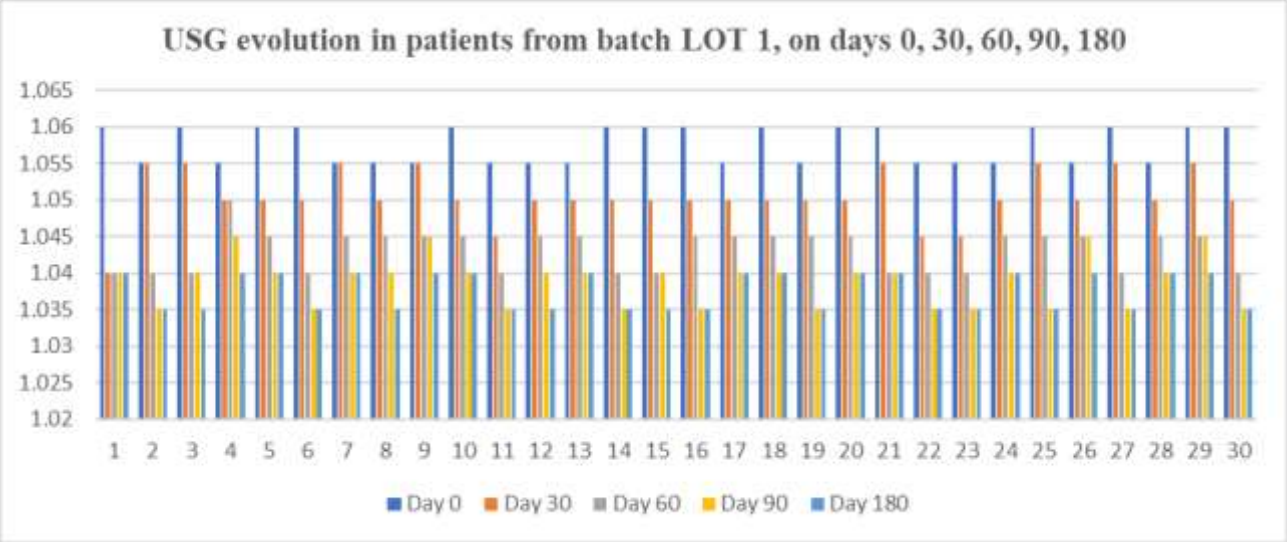

Figure 4. Graphical representation of the evolution of urine density (USG) of all cases that are part of batch LOT 1 (n = 30), on the day of presentation (day 0), at one month (day 30), at two months (day 60), at three months (day 90) and 6 months (day 180) days.

Table 1.

Evolution of UPC of all cases that are part of batch LOT 1 (n = 30), on the day of presentation (day 0), at one month (day 30), at two months (day 60), at three months (day 90) and 6 months (day 180) days.

PATIENT NO.	PATIENT	DAY 0	DAY 30	DAY 60	DAY 90	DAY 180
P1	M, 5 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P2	M, 6 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P3	M, 4 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P4	F, 9 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P5	M, 7 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P6	M, 1 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P7	M, 8 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P8	F, 9 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P9	M, 4 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P10	M, 1 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P11	M, 3 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P12	F, 7 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P13	M, 2 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P14	M, 9 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P15	F, 10 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P16	M, 3 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P17	M, 5 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P18	M, 1 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P19	F, 8 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P20	M, 4 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P21	M, 2 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P22	M, 8 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P23	M, 3 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P24	F, 9 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P25	M, 6 YO	>0.2	>0.2	>0.2	<0.2	<0.2

P26	M, 4 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P27	F, 10 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P28	M, 2 YO	>0.2	>0.2	>0.2	<0.2	<0.2
P29	M, 5 YO	>0.2	>0.2	<0.2	<0.2	<0.2
P30	M, 2 YO	>0.2	>0.2	>0.2	<0.2	<0.2

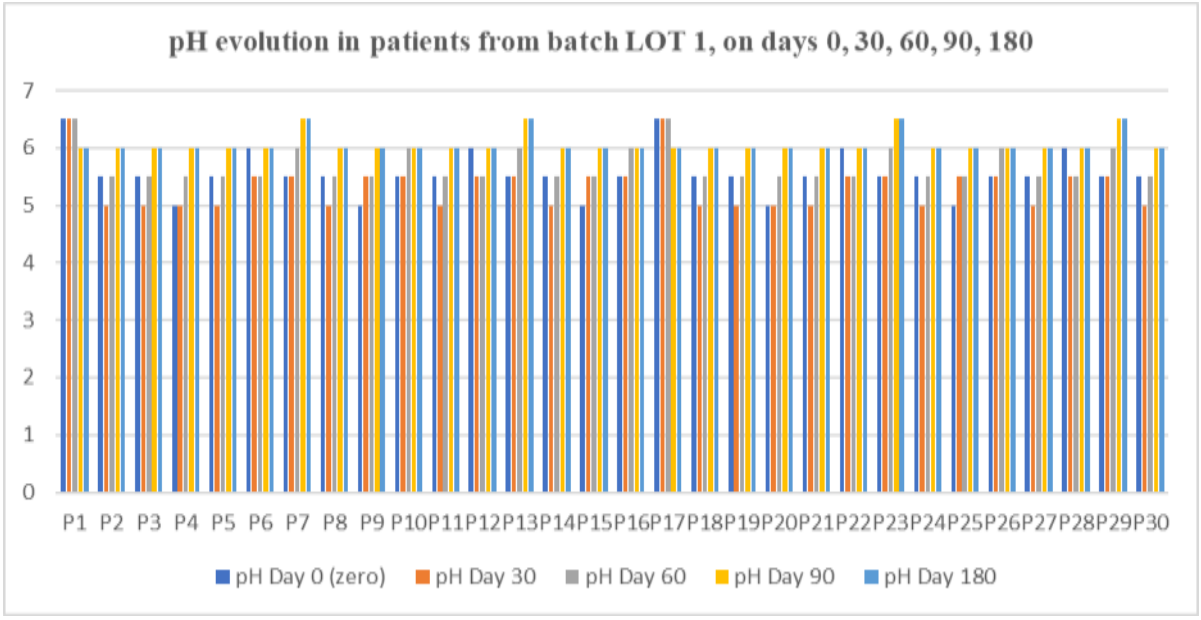

Figure 5. Graphical representation of the evolution of urinary pH of all cases that are part of batch LOT 1 (n = 30), on the day of presentation (day 0), at one month (day 30), at two months (day 60), at three months (day 90) and 6 months (day 180) days.

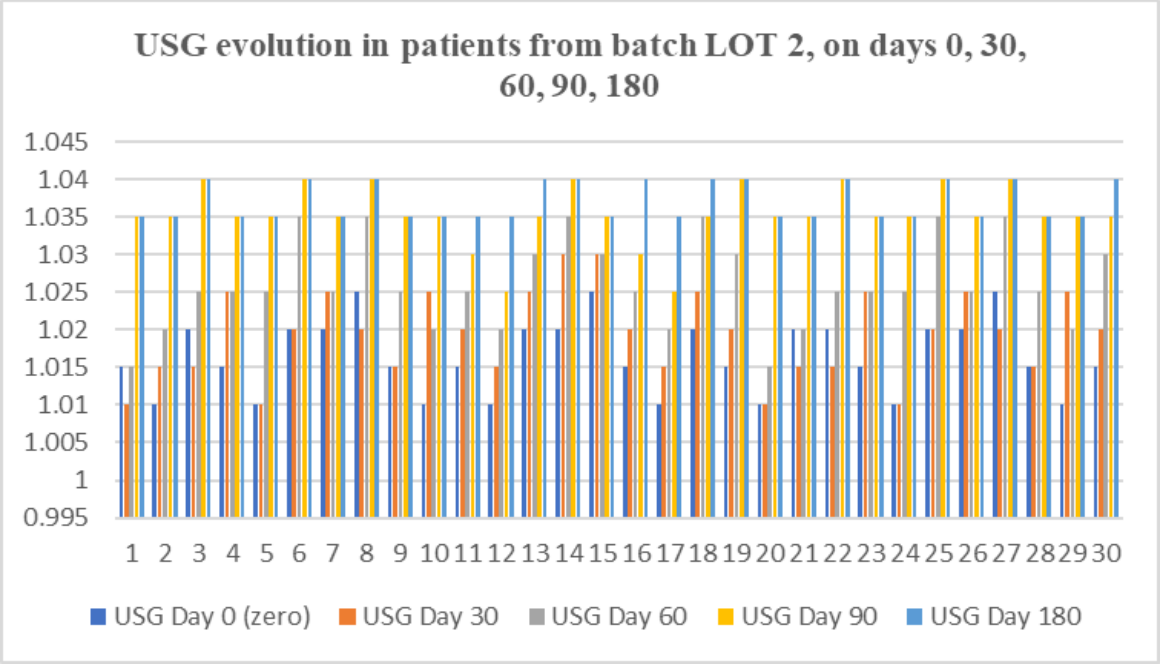

Figure 6. Graphical representation of the evolution of urine density (USG) of all cases that are part of batch LOT 2 (n = 30), on the day of presentation (day 0), at one month (day 30), at two months (day 60), at three months (day 90) and 6 months (day 180) days.

Table 2.

Evolution of UPC of all cases that are part of batch LOT 2 (n = 30), on the day of presentation (day 0), at one month (day 30), at two months (day 60), at three months (day 90) and 6 months (day 180) days.

PATIENT NO.	PATIENT	DAY 0	DAY 30	DAY 60	DAY 90	DAY 180
P1	M, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P2	M, 6 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P3	M, 4 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P4	F, 10 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P5	M, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P6	M, 1 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P7	M, 8 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P8	F, 7 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P9	M, 4 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P10	M, 2 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P11	M, 5 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P12	F, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P13	M, 2 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P14	M, 8 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P15	F, 8 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P16	M, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P17	M, 4 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P18	M, 2 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P19	M, 7 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P20	M, 5 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P21	M, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P22	M, 2 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P23	M, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P24	F, 9 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P25	M, 7 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P26	M, 4 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P27	F, 18 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P28	M, 3 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P29	M, 4 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2
P30	M, 1 YO	0.2 - 0.4	0.2 - 0.4	<0.2	<0.2	<0.2

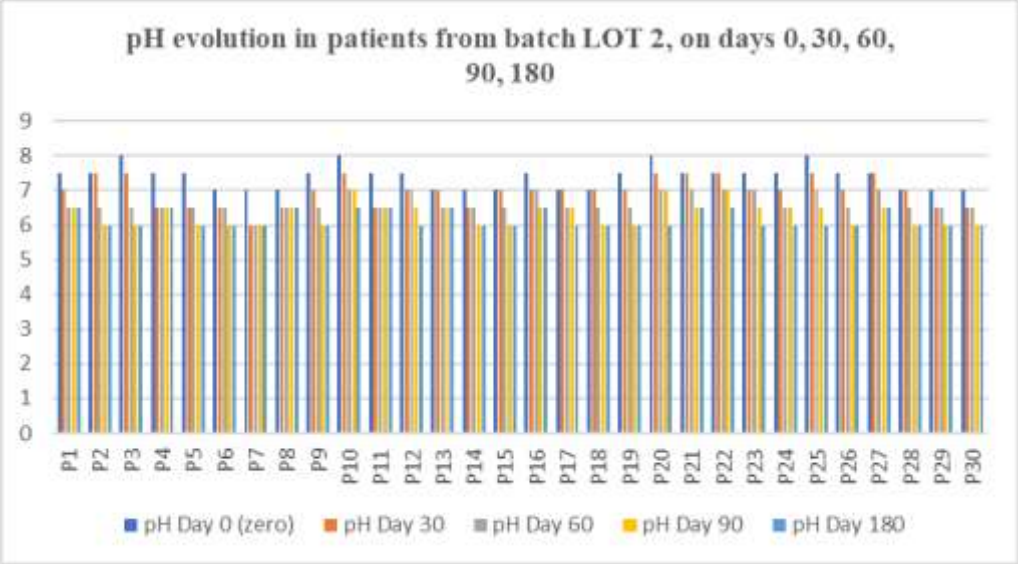

Figure 7. Graphical representation of the evolution of urinary pH of all cases that are part of batch LOT 2 (n = 30), on the day of presentation (day 0), at one month (day 30), at two months (day 60), at three months (day 90) and 6 months (day 180) days.

CONCLUSIONS

Hydration is an essential component in the management of patients diagnosed with feline urological syndrome.

Administering an alkaline hydric diet in patients with aciduria is a solution to counteract the effect of metabolism on urinary pH.

Potassium supplementation in polyuric patients is a beneficial solution in hypokalemia therapy.

Potassium restriction in oliguric patients is a beneficial solution in the therapy of hyperkalemia.

Monitoring the pH, USG and UPC is mandatory in adapting the therapy of patients diagnosed with feline urological syndrome.

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THE PREVALENCE OF INTESTINAL PARASITES IN DOGS FROM SHELTERS IN CONSTANȚA COUNTY-ROMANIA

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Abstract

This study was designed to evaluate the prevalence of gastrointestinal parasites in shelter dogs from Constanța, a south-eastern county from Romania. In order to assess, in 2022 and 2023, individual and pooled fecal samples, were collected from 611 dogs from 9 shelters. Fecal samples were examined using standard flotation and sedimentation methods. The overall prevalence of gastrointestinal parasites was 74.63%. Eggs of hookworms (*Ancylostoma* sp. and *Uncinaria* sp.) were the most frequently detected (55.97%), followed by *Cystoisospora* sp. (31.91%), *Trichuris vulpis* (27.33%), *Toxocara canis* (21.27%), %), *Capillaria* sp. (2.78%) and *Toxascaris leonina* (1.96%). Cases of single infestation were found in 217 (35.51%) of the tested dogs. Mixed infestations with two or more species of parasites were observed in 239 samples, representing 39.11% of the total samples analyzed. These results will be useful for establishing health care programs in dog shelters and for implementing effective strategies in controlling the intestinal parasites, in order to restrain the spread of zoonotic parasites and to raise awareness of their impact on public health.

Key words: shelter dogs, gastrointestinal parasites, prevalence

Animal shelters play an important role in human and animal health, therefore veterinary monitoring of livestock can prevent possible epidemiological outbreaks.

Given the fact that Romania ranks 6th in Europe for the total amount of dogs according to "Statista", which in relation to the number of inhabitants, places us in the lead with a number of more than 200 dogs per thousand inhabitants, further studies on the epidemiology of parasitic diseases in stray animals are urgently needed. Although Romanian law requires local authorities to have a service for managing stray dogs and through it, community dogs to be collected from the public domain and placed in specialized shelters, where, if they are not adopted within 14 days, they should be euthanized, in Constanța county euthanasia is not an option, and the captured dogs are identified, dewormed, vaccinated, sterilized and ready for adoption. Because in Constanța county there are only four public shelters, and the number of the stray dogs is increasing, a lot of improvised shelters apeared, in order to protect the stray dogs and to give them a proper life, but even if they are well-intended, these shelters do more harm than good to public health, ignoring the general methods of prevention in veterinary medicine.

Given that in recent years the number of adoption applications has been decreasing, most shelters are overcrowded, and the animals face various stressors (Tuber *et al*., 1999). All these factors are leading to parasitic contamination of the environment and the easy spread of parasitic infections, which further lead to an increased risk of zoonotic diseases in the human population and a strong impact on public health.

Because shelter dogs live in close cohabitation and poor hygienic conditions, the parasitism may pose a serious problem (Tamara *et al.*, 2021). A contaminated environment is conducive to parasite transmission in dogs, and the major pathogens that cause illness in shelter dogs are those transmitted by ingestion of parasites in different stages of development. (Ortuno *et al.*, 2011).

The occurrence of gastrointestinal parasites is variable and depends on many factors, such as age, living conditions, health status of the animal, diagnostic techniques used, and region studied

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(Táparo *et al.*, 2006**;** Mircean *et al.*, 2017**;** Lara-Reyes *et al.*, 2021).

MATERIAL AND METHOD

Constanța is a county located in the south-east of Romania, being bordered to the east by the Black Sea, to the west by the Lower Danube and to the north by Tulcea County. It has a surface area of 7.104 km2 and a total population of 573,331 inhabitants.

The climate of Constanța is continental, and the influences of the Black Sea are felt in the cold season, the temperatures remaining above the freezing point during the warm season, the climate being affected by the sea breeze. The average annual temperature varies between 9.9 ^{o}C and 11.7^{o}C. Multiannual average precipitation varies from 261 mm at Sulina station to 488 mm at Cernavoda (Maftei et al, 2008).

The study was conducted between 2022 and 2023, in nine dog shelters, of which three were public, two private and four were improvised shelters, located in Constanța County. The public shelters were in Constanța city, (n=271), Hârșova city (n=47) and in Năvodari city (n=20). The private shelters were one in Cernavodă city (n=50) and one in Techirghiol city (n=42), and the improvised shelters were in Ovidiu city (n=49), and the other three in Nicolae Bălcescu village (n=33), Runcu village (n=72) and Peninsula village (n=27). The four improvised shelters are isolated, situated on the outskirts or outside the villages. Two of the shelters have all the animals kept together, free, without having limited access, another has all the dogs kept in bunk cages in a garage. The last improvised shelter, from Runcu, has 2/3 of the dogs kept in closed pens without light, gathered two or three together in a pen and 1/3 of the dogs are kept free in the yard.

In order to carry out our research, individual and pooled fecal samples, were collected from 611 dogs from 9 shelters situated in Constanța County during the study period.

The samples were collected from dogs which had spent 2 months or more in the facility.

Fecal specimens were placed in screw-topped containers with uniquely labeled identification number and date of collection. Data on sex, age, status, were also recorded.

Fecal samples were processed by direct smear for detection of motile trophozoites or cysts of protozoa, flotation technique, using saturated sodium chloride for extraction of lighter helminth eggs and coccidian oocysts or sporocysts and sedimentation technique for recovering heavier helminth eggs.

RESULTS AND DISCUSSIONS

The results showed that more than half of the dogs (456/611) were infected with at least one species of gastrointestinal parasites, and the overall prevalence of GI parasites was 74.63 %.

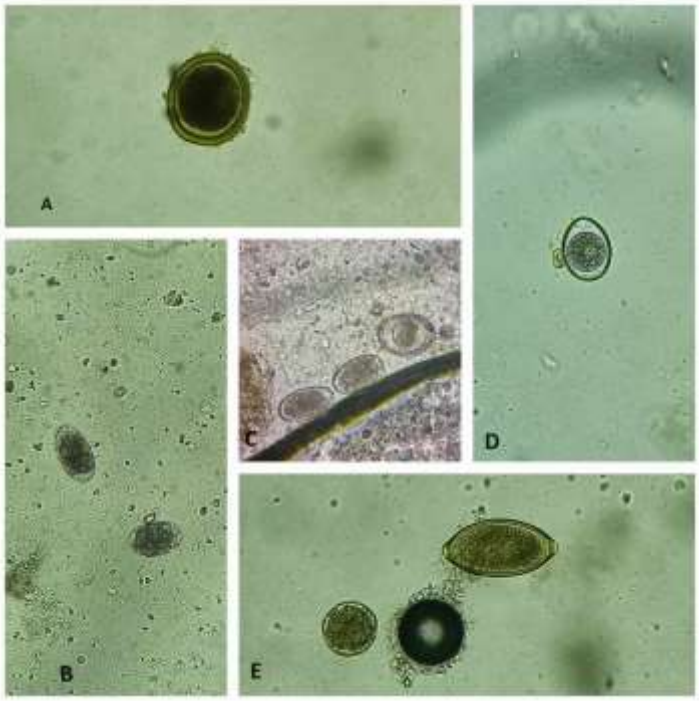

Figure 1. **Eggs, cysts and oocysts of intestinal parasites, in fecal samples from stray dogs from Constanța County. (A) Egg of *Toxocara* spp., (B) Egg of *Ancylostoma* spp., (C) Egg of *Toxascaris leonina*, (D) Oocyst of *Cystoisospora* spp., (E) Egg of *Trichuris vulpis***

Numerous studies have had similar results, indicating a parasite presence of 67.1% (Ursache *et al.*, 2016) and 66.6% (Mircean *et al*., 2017) in dogs in Romania, 81.4% in Palestine in stray dogs and 48.4% in pets (Othman *et al.*, 2021), 75.4% in Serbia (Sommer *et al.*, 2017) and 58.3% (Ilic *et al.*, 2021), 63.5% (148/233) in northern Spain (Regidor-Cerrillo *et al*., 2020), between 48.1% and 64.9% in Croația (Brezak *et al.*, 2017) and 32.2% also in Croatia (Faraguna *et al*., 2023), 64.5% in Bulgaria (Iliev *et al*., 2020), 54.05% in dogs from Romania (Soran *et al*., 2020), 37.2% in Mexico (Reyes *et al*., 2021), 26 % in Greece (Papapzahariadou *et al*., 2007), 30.4 % in Turkey (Senlik *et al*., 2006) and 40.7 % in Albania (Shukullari *et al*., 2015).

Ancylostoma sp./Uncinaria sp. eggs were the most frequently observed in feces samples (55.97 %), followed by *Cystoisospora* sp. (31.91%), *T. vulpis* (27.33 %), *Toxocara canis* (21.27 %), *Capillaria* sp. (2.78%), *Toxascaris leonina* (1.96%) and *Demodex* (0.49%) (*Table 1).*

Even though it is not the subject of this study, following the examination of fecal samples, the adult mite *Demodex canis* was also identified.

Table 1

Parasite species	Number of parasite species in infected dogs/shelter type											Total Prevalence
	Public shelter n=338				Private shelter n=92		Improvised shelter n=181					
	Constanța n=271	Hârșova n=47	Năvodari n=20	Total prevalence Public shelters n=338	Cernavodă n=50	Techirghiol n=42	Nicolae Bălcescu n=72	Ovidiu n=49	Runcu n=72	Peninsula n=27	Total prevalence Improvised shelters n=181	
Ancylostoma/ Uncinaria sp.	121	47	20	55.62%	0	0	33	49	72	0	85.08%	55.97%
Trichuris vulpis	18	47	5	20.72%	0	0	33	49	15	0	53.59%	27.33%
Toxocara canis	42	47	5	27.81%	0	0	0	0	36	0	19.89%	21.27%
Isospora canis	115	47	0	47.92%	0	0	33	0	0	0	18.23%	31.91%
Toxoascaris leonina	0	0	0	0%	0	0	0	0	12	0	6.63%	1.96%
Capillaria sp.	0	17	0	5.02%	0	0	0	0	0	0	0%	2.78%
Demodex	0	3	0	0.88%	0	0	0	0	0	0	0%	0.49%

The prevalence of parasitic elements

In our study, *Ancylostoma/Uncinaria* is the most common enteric pathogen, being found in the stool samples of 55.97% of the studied dogs. A similar result was also obtained in Bulgaria, 54.1% (Iliev *et al*., 2020), 29.53% in Brazil (Souza *et al*., 2023), 8.00% in Palestine (Othman *et al*., 2021), and in Romania 33% (Mircean *et al*., 2017） and 10.4% (Ursache *et al*., 2016).

The prevalence obtained for *Trichuris vulpis* is 27.33%, a result comparable to other studies carried out in Romania, 25% (Mircean *et al*., 2017), 20% (Ursache *et al*., 2016), in Bulgaria 15.1% (Iliev *et al*., 2020) and respectively 10.42% (Iliev *et al*., 2017) and 7.7% in Serbia (Ilic *et al*., 2021).

In the case of protozoa, their prevalence was 31.75%, being entirely represented by *Isospora canis*, while other studies from Romania showed a positivity of 11.9% for protozoa, of which 8.57% was for *Isospora canis* (Soran *et al*., 2020), or 16.1% (Ursache *et al*., 2016). In Serbia the prevalence is 8% (Ilic *et al*., 2021) and 4.1% in Bulgaria (Iliev *et al*., 2020).

The prevalence we found for *Toxocara canis* (21.27%), is lower compared to Palestine, 46.0% (Othman *et al*., 2021) or other studies from Romania, in dogs with owners where it is 34.8% (Ursache *et al*., 2016), but higher than in Bulgaria, where a prevalence of 6.4% was recorded (Iliev *et al*., 2020) or in Brazil, where the prevalence is 7.52% (Souza *et al*., 2023) and Serbia where it is 18.5% (Ilic *et al*., 2021).

Following a study using published data from 26 European countries over the past 25 years, the average European prevalence for *Toxocara canis* was 14.6% in dogs (Overgaauw *et al*., 2020).

Cases of single infestation were found in 217 (35.51 %) of the sampled dogs.

Mixed infestations with two or more parasite species were observed in 239 samples, representing 39.11% of the total analyzed samples (*figure 2*).

The most frequently detected co-infestations were with *T. vulpis/Cystoisospora* sp./*Ancylostoma* sp. (10.31%) and *T. vulpis /Ancylostoma* (8.83 %) (*Table 2*) .

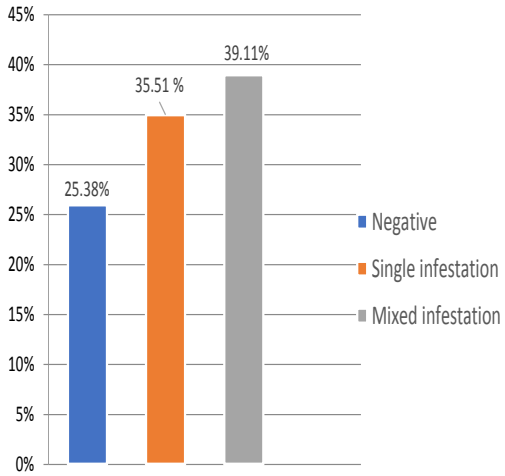

Figure 2 The prevalence of single and mixed infestation in sampled dogs

Table 2

Cases of mixed infestations (n=611)

Coinfestation	Positive (%)
T. canis + *Ancylostoma/Uncinaria* sp.	14 (2.29)
T. vulpis + *Ancylostoma/Uncinaria* sp.	54 (8,83)
T. canis + *Cystoisospora* sp.	24 (3,92)
Cystoisospora spp.+ *Ancylostoma/Uncinaria* sp.	22(3.6)
T. vulpis + *Ancylostoma/Uncinaria* sp.+ *Cystoisospora* sp.	33 (5.4)
T. vulpis + *T. canis* + *Cystoisospora* sp.	18 (2.94)
T. canis + *Ancylostoma/Uncinaria* sp + Toxascaris leonina	12 (1.96)
T. canis + *Ancylostoma/Uncinaria* sp + *T. vulpis*	15 (2.45)
T. canis + *Ancylostoma/Uncinaria* sp. + *Cystoisospora* sp+ *T. vulpis*	30 (4.90)
T. canis + *Ancylostoma/Uncinaria* sp. + *Cystoisospora* sp. + *Capillaria* sp.+ *T. vulpis* + *Demodex*	3 (0,49)
T. canis + *Ancylostoma/Uncinaria* sp. + *Cystoisospora* sp. + *Capillaria* sp.+ *T. vulpis*	14 (2,29)

After examining the samples taken from the 4 improvised shelters, we found that in the case of two shelters, where the dogs were kept free in a fenced area, the results of the copro-parasitological

examinations were identical for all the canine population present in the shelters, and the identified parasitic elements were common in the entire dog population (*Table 3*).

Table 3

The prevalence of parasitic elements in improvised shelters

Parasite species	OVIDIU SHELTER		N.BĂLCESCU SHELTER		RUNCU SHELTER		PENINSULA SHELTER		Total Prevalence n=181
	Positive samples/ collected samples	P%	Positive samples/ collected samples	P%	Positive samples/ collected samples	P%	Positive samples/ collected samples	P%	
Ancylostoma/ Uncinaria	**49/49**	**100%**	**33/33**	**100%**	**72/72**	**100%**	**0/27**	**0%**	**85.08%**
Trichuris vulpis	**49/49**	**100%**	**33/33**	**100%**	**15/72**	**20.83%**	**0/27**	**0%**	**53.59%**
Isospora canis	**0/49**	**0%**	**33/33**	**100%**	**0/72**	**0%**	**0/27**	**0%**	**18.23%**
Toxocara canis	**0/49**	**0%**	**0/33**	**0%**	**36/72**	**50%**	**0/27**	**0%**	**19.88%**
Toxasscaris leonina	**0/49**	**0%**	**0/33**	**0%**	**12/72**	**16.66%**	**0/27**	**0%**	**6.62%**

Analyzing the table content, we can see a very high prevalence, of 85.08%, for *Ancylostoma/Uncinaria* sp., followed by *T. vulpis* (53.59%), *Toxocara canis* (19.88%), *Isospora canis* (18.23%) and *Toxascaris leonina* (6.62%), which denotes the fact that these improvised shelters represent a great danger to public health with a serious impact on the population.

Within the study, an evaluation of the protozoa prevalence in the public shelter from Constanta was also carried out, regarding the type of shelter flooring.

Despite the few published data, the floor covering of shelters is known as a factor of parasitic infection in animal shelters.

In our study, the type of shelter flooring was found to be relevant for the epidemiology of species, particularly for protozoan infections. A higher prevalence of protozoa infestation was found in dogs kept without concrete floor and drainage (56%), compared with the dogs that were kept on concrete floor 35% (*Table 4*).

A similar result was obtained in Romania (Soran *et al*, 2020), a higher prevalence for protozoa infestation being found in dogs kept without concrete (6.56%), compared to the dogs kept in shelters with concrete floor (4.76%).

Table 4

The prevalence of protozoa related to the type of flooring in Constanța Public Shelter

Parasite species	MDF Flooring		Concrete floor	
Isospora canis	51/91	(56%)	63/180	(35%)

No significant differences were found in prevalence of different parasite species in males and females, results being in agreement with other studies. Additionally, other factors such as season may impact the appearance and frequency of cysts in the stool.

CONCLUSIONS

The results of this study show a high prevalence of digestive parasitic diseases in stray shelter dogs from Constanța County, underlining the need to establish proper monitoring and control

programs for parasitic elements through a rigorous shelter sanitation and correctly applied deworming.

Stray dogs represent a permanent reservoir of pathogens that are directly transmitted in nature through feces. That is why studies on the prevalence of digestive parasites are essential and must be carried out in a sustained manner.

The epidemiological investigation of the main digestive parasitosis found in stray shelter dogs in Constanța County represents an essential and complex approach in establishing the risks involved in parasitic zoonoses.

The high prevalence for intestinal parasitic co-infections in dogs reported here, creates real concerns and exponentially increases the degree of infestation among people. Therefore, in order to prevent possible zoonotic outbreaks, deworming must be done more often and more responsibly.

A big red flag are the improvised shelters, which are not regulated by law and play an important link in the zoonotic chain. The results obtained are high enough to worry us and to make us take measures to limit the spread of parasitic diseases.

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PREVALENCE OF INFECTION WITH *DIROFILARIA IMMITIS* AND *DIROFILARIA REPENS* IN DOGS FROM THE SOUTH-EASTERN PART OF ROMANIA

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As a result of global warming, the change in the biological cycle of vectors and the increase in intercontinental travels, we are now facing an increase in the number of cases of vector-borne diseases. Among these, heartworm disease has escalated in recent years in Romania, with cases increasing in many parts of our country. Our study was carried out in the South-Eastern part of Romania, with the aim of establishing the prevalence of heartworm disease in dogs, with the identification of the two species *Dirofilaria immitis* and *Dirofilaria repens*. Blood samples were collected during the period May - July 2022 and January - July 2023, from 220 dogs with and without an owner from Tulcea county, from places such as Tulcea, Murighiol, Somova, Mineri, Niculițel. The blood samples were tested through the Knott method, which is considered as the reference method in the diagnosis of dirofilariosis in dogs. The results show a prevalence of *Dirofilaria spp.* infection of 11.36% in dogs. Of the 220 samples examined by the Knott test, 25 were positive for circulating microfilariae, of which 21 showed infestations with *Dirofilaria immitis* and 4 with co-infection with *Dirofilaria immitis* and *Dirofilaria repens*. The study underlines the importance of introducing monitoring and control programs for heartworm disease in dogs in Romania.

Key words: dirofilariosis*;* dogs; Tulcea;

Introduction. Canine cardiopulmonary and subcutaneous dirofilariasis are caused by zoonotic filaroid worms, *Dirofilaria immitis* and *Dirofilaria repens*, respectively. Human dirofilariasis in the ancient world is primarily caused by *D.repens*, whereas *Dirofilaria immitis* is highly significant for veterinary purposes. Numerous mosquito species, including those of the genera Culex, Aedes, Ochlerotatus, Anopheles, Coquillettidia, Armigers, Ansonia and Psorophora, have been identified as competent vectors of these mosquito-borne filarial infections. These mosquito-borne filarial infections share the same definitive hosts, which are primarily canids (Younes *et al*., 2021).

Historically, canine heartworm infection was restricted to southern Europe. Up until the mid-1980s, northern Italy was the most endemic region for the parasite. Birago discovered the parasite in a greyhound dog during an autopsy in northern Italy in 1626 (Genchi *et al*., 2014).

Dogs that are infected are the primary source of *D. repens* because their peripheral blood typically contains microfilariae. *D. repens* infections have been documented as widespread in central and southern Italy as well as in other southern European nations like France, Greece and other former Yugoslavia since the early 1900s (Genchi and Kramer, 2017).

The first known human observation of *D.repens* was probably made in 1566 by the Portuguese physician Amato Lusitano, who noticed the tip of a worm suddenly appearing in the big angle of the eye in a 3-year-old girl. The worm is sometimes located in the eye, causing opacity (Capelli *et al.*, 2018).

As for the present, both the quantity of epidemiological reports and the epidemiological state have altered. Climate change has resulted in new climatic conditions for Europe, which affects the presence of new competent vectors that broaden the risk zone (mosquitoes more resistant to low temperatures, overwintering eggs, etc.) and increase the duration of exposure to the parasite (daytime and crepuscular/nocturnal mosquitoes) (Morchón *et al.*, 2022).

Heartworm illness is linked to numerous pathophysiological pathways, such as right ventricular overload and cardiopulmonary abnormalities linked to parenchymal lung disease (Tudor *et al.*, 2014).

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Heartworm disease in dogs can present with a wide range of symptoms, from mild to severe and even fatal. Reduced exercise tolerance, a persistent cough that advances from moderate to severe dyspnea, prostration, ascites, cachexia, and post-exercise syncope or excitation are the most common early clinical symptoms (Bendas *et al.*, 2022).

The second species, *Dirofilaria repens* causes some common signs in pets, like itch (pruritus), papulae, erythema, alopecia, crusting, hyperkeratosis, lichenification and acantosis. Occasionally, subcutaneous nodules can be seen, formed by a cyst enclosing an adult nematode. In most cases, however, no pathogenic signs are observed in animals carrying *repens* microfilariae (Tarello, 2011).

A minimum of ten years ago, *Dirofilaria spp*. were identified in Romania. According to Tomazatos *et al*. (2018), there have been different investigations on the prevalence of parasites in dogs that have found local infection rates ranging from 3% to over 60%.

The aim of this study was to obtain a current prevalence of infection with *Dirofilaria immitis* and *Dirofilaria repens* in dogs from the south-eastern part of Romania.

MATERIAL AND METHOD

This study included 220 dogs from Tulcea county, Romania. Their age and gender were taken into account. Based on this data, a statistic was made, resulting in 124 females and 96 males with the age between 7 months and 18 years. Regarding the medical history of the dogs, it was known in only 55 of the dogs , since the rest of the samples came from dogs without an owner which did not have any medical record. Based on the age, the dogs were grouped as follows: puppies (7 months – 1 year); young (1-3 years); adults (4-7 years); seniors (8-10 years); and geriatrics (11-18 years). Therefore, 220 blood samples were collected in tubes with anticoagulant (ethylenediaminetetraacetic acid- EDTA) from the cephalic vein. The samples were analyzed in the Clinical Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine in Iasi.

Laboratory techniques

Blood samples were tested for the identification of circulating microfilariae using the modified Knott method.

The modified Knott's test is an easy and inexpensive technique based on concentration, staining, detection and morphometric identification of circulating of different species. The technique foresees the dilution of 1 mL of EDTA venous blood with 9 mL of 2% formalin (Genchi *et al*., 2021).

The technique consists in adding to a 15 ml tube, 1 ml of canine blood sample, which was mixed with 9 ml of 2% formalin. The tube was centrifuged for 5 minutes at 1500 rpm and the supernatant was removed, adding 1% methylene blue to the sediment. After mixing, from the resulting sediment, 1 drop was transferred to a slide, covered with a coverslip and examined under an optical microscope.

The modified Knott test (Knott, 1939) is preferred and is the recommended test for circulating microfilariae (Evans *et al*., 2019).

RESULTS

The 220 samples come from 124 females and 96 males.

Of the 198 samples collected in Tulcea, 55 samples come from dogs with owners and 143 from dogs without owners.

From the 143 blood samples collected from stray dogs, the vast majority of samples were collected from dogs from the Public Shelter and thus resulted in 128 negative samples, 11 positive, infected with *Dirofilaria immitis* and 4 positive with co-infection with *Dirofilaria immitis* and *Dirofilaria repens (figure 1).*

Figure 1 – *Dirofilaria* spp. – ob. X 10

And out of the total of 55 samples from dogs with owners, 48 were negative and 7 were infected with *Dirofilariasis immitis.*

In the town of Niculițel, 19 blood samples were collected, of which 16 were negative and 3 were positive for *Dirofilaria immitis.*

The rest of the samples, in total of 3 collected from dogs from Murighiol and Somova, and turned out to be negative.

In terms of age, the vast majority of blood samples were collected from young dogs, aged between 1 and 3 years, as well as from adult dogs, aged between 4 and 7 years (76,8%) (*table 2*).

Table 1

Prevalence of *Dirofilaria immitis* and *Dirofilaria repens* in the localities of Tulcea county

Localities	Positive samples (Total positive/Total samples)	
Tulcea	22/198	(11.11%)
Niculițel	3/19	(15.78%)
Somova/Murighiol	0/3	(0%)

Of the 124 females tested, 112 were negative and 12 were positive (9.67%). Of the 12 samples, 11 were positive for *Dirofilaria immitis* and 1 for co-infection with *Dirofilaria immitis* and *Dirofilaria repens* (*table 1*).

Of the 96 males tested, 83 were negative and 13 were positive (13.54%). Of the 13 samples, 10 were positive for *Dirofilaria immitis* and 3 for *Dirofilaria immitis* plus *Dirofilaria repens* (*table 2*).

Table 2

The results of the samples analyzed by gender and age

Gender	Positive samples (Total positive/total tested)	
Female	12/124	(9.67%)
Male	13/96	(11.36%)
Age		
1-3 years	7/129	
4-7 years	13/64	
8-10 years	4/21	
11-18 years	1/6	

Thus, the modified Knott test showed a total of 25/220 samples positive for microfilariae (11.36%), of which 21/25 samples were positive for *Dirofilaria immitis* microfilariae and 4/25 for *Dirofilaria immitis* plus *Dirofilaria repens.*

The highest prevalence was detected in the age group 4-7 years, followed by the groups 1-3 years (young), 8-12 years and geriatrics dogs (13-18 years).

Table 3

Prevalence of *Dirofilaria immitis* and *Dirofilaria repens*

Species	***Positive samples*** *(Total positive/Total samples)*	
Dirofilaria immitis	21/220	(9.54%)
Dirofilaria repens	0/220	(0%)
D. immitis and *D. repens*	4/220	(1.81%)

DISCUSSIONS

This study presents the results obtained from the examination of blood samples of 220 dogs from Tulcea county using parasitological methods, in order to establish the prevalence of canine dirofilariosis in this county.

Analyzing various studies from previous years, we can observe an increased prevalence of heartworm disease in the South-East of Romania. As can be seen in an article written in 2015 in

which it is specified: Based on molecular detection, an overall prevalence of 6.92 % ($n = 27$; 95 % confidence interval (CI) 4.70–10.03 %) for *D. repens*, 6.15 % ($n = 24$; 95 % CI 4.07–9.14 %) for *D. immitis* and 2.05 % ($n = 8$; 95 % CI 0.96–4.16 %) for *A. reconditum* was recorded, with significant variations according to sampling areas. Coinfections of *D. immitis* and *D. repens* were recorded in 23.91 % ($n = 11$) positive dogs (Ionică et al., 2015). Comparing the number of blood samples analyzed and the positive results, we can see an increase in the prevalence of heartworm disease.

No study has been published on risk factor analyzes using a multivariate approach, which would be more suitable for highlighting confounding factors and biases (Capelli *et al*., 2018). Therefore, some of the associations found and often reported as risk factors are likely the results of the interaction of different factors related to the host (sex, age, bread and lifestyle), the vector (presence, density, vectorial capacity and attraction to dogs), the environment (rural, urban, climate) and the human intervention (use of specific chemoprophylaxis and/or physical or chemical protection against mosquitoes) (Capelli *et al.*, 2018).

Comparing these factors with the data presented, it was found that the most increased prevalence was among males (13 positive samples), males classified as adults, with age between 4-7 years.

From the total number of 220 samples, 25 were positive for circulating microfilariae, of which 21 were infected with *Dirofilaria immitis* and 4 with co-infection with *Dirofilaria immitis* and *Dirofilaria repens.* In the specialized literature it is noted that the interspecific relationship between *D. immitis* and *D. repens* has been only partially studied, suggesting an inhibition of the development of *D. immitis* in dogs previously infected with *D. repens (*Ionică *et al.*, 2017). However, it indicates that once the animal develops a patent co-infection, the microfilariae of the two species show a similar circadian periodicity, probably as a reaction to the same stimuli, without apparent influences between them (Ionică *et al.*, 2017).

Referring to *Table 3*, where the prevalence of heartworm disease is presented in some of the localities in Tulcea county, we notice that it is higher in rural areas (Niculițel: 15.78%). This difference appears in general due the lack of information about externalparasites treatments , but global warming should not be omitted, as it is mentioned in the article written by Martinescu *et al.*, 2022. In this article it is specified that is possible that Global warming is influencing the increase in cases of this disease, according to the abundance of etiological agents (Martinescu *et al.*, 2022).

CONCLUSIONS

The prevalence that we established in dogs (11.36%) of dirofilariosis in the South-East of Romania, respectively in Tulcea county, has an alarming increase, thus monitoring the disease, as well as prophylactic treatment is a main objective. Also, clinician veterinarians should include this disease in the differential diagnosis of various cardiac, respiratory and dermatological diseases.

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FOOD SAFETY IN THE AGE OF TRANSPARENCY: CLEAN LABEL PRODUCTS IN THE POST-COVID-19 ERA

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Abstract

Clean-label products are defined as foods and beverages formulated with simple, natural, and familiar ingredients, instead of using artificial ingredients and additives. The clean label movement has gained popularity because of a rise in consumer demand for authentic, transparent, and healthier foods and beverages. In the post-COVID-19 era, the clean label trend has evolved considerably, indicating a shift in consumer preferences and demands. The COVID-19 pandemic has had a significant effect on consumer behavior, particularly regarding food safety and products with clear labels. According to recent studies, during the pandemics, consumers have shown an increased interest in products with clean labels as they seek healthier choices. In addition, the pandemic has impacted consumer purchasing patterns, with a shift toward ingredient examination and an increased demand for healthful products. In conclusion, the COVID-19 pandemic has highlighted the significance of the clean label trend, redirecting consumers toward safe, natural, and transparent food products, and emphasizing the importance of local purchasing and sustainable supply chains.

Key words: clean-label, COVID-19, food safety, consumer.

In recent years, there has been an increase in interest among consumers in food safety and a heightened awareness of health concerns associated with food products. The issues for ensuring safe and healthy food for the public include the growing use of improper ingredients and the related misleading strategies in packaging and labeling.

A shift in the preferences of consumers has led to a significant effort in the food sector to avoid particular ingredients by reformulation (Storm S., 2015). Ingredients commonly selected for removal include those that are artificially generated (artificial flavors or Red 40) and possess complex, "chemical sounding" terms (methyl crystalline cellulose carrageenan) (Berenstein N., 2018). While regulatory bodies consider these ingredients to be safe, the customers view them as potentially dangerous due to their unfamiliarity and their perspective of chemicals as dangerous to their health (Moskowitz H.R. et al., 2012; Wansink B. et al., 2014; Maruyama S. et al., 2021).

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Ingredion (2014) as referenced by Asioli D. et al., 2017, states that when a product is designated being clean, it means that it can be advertised as *"natural," "organic," and/or "free from additives/preservatives"* Based on the same study, food producers can use a clean label approach by utilizing ingredients that can be easily identified by customers, such as those often seen in their kitchen cupboards. The ingredients list should be clear, concise, and mostly comprise less processed foods, if possible. Avoiding using names that resemble chemicals or e-numbers is also considered for this type of product.

The ingredients list needs to be concise, uncomplicated, and mostly consist of foods that have been minimally processed if applicable. The inclusion of names resembling chemicals or e-numbers shall be avoided. According to Edwards (2013) cited by Asioli D. et al., 2017, a clean label is characterized by the absence of chemical additions, clear and comprehensible ingredients list, and production methods that adhere to traditional practices with minimal processing.

The health consciousness of customers influences their attitudes towards food consumption, as well as their intents to purchase a certain product. Research indicates that those who prioritize their health and possess a higher level of understanding of nutrition are more inclined to pay attention to the ingredients on food labels (Cavaliere A. et al., 2017; Chang M.Y. et al., 2022). Also, following the impact of the COVID-19 crisis on the food ingredient business, there is an increase in demand for products with clean labels as consumers seek out healthier and more reliable options. Thus, this review aims to discuss recent research in the food sector pertaining to clean label foods, while considering how the COVID-19 pandemic has impacted consumer preferences based on generational differences.

CONSUMERS PURCHASING PATTERNS

In a study conducted by Lee W.I. et al. (2017), it was discovered that product aspects, which includes information about the product, its quality, and pricing, have a positive influence on a consumer's decision to make a purchase. Moreover, purchase intention is seen as a preliminary factor to the actual act of buying (Fishbein M., 2011). Purchase intention is the likelihood that a buyer will choose to buy a particular product (Chang M.Y. et al., 2022).

A greater inclination to purchase indicates an increased probability of making a buying choice (Angelis M.D. et al., 2017). Moreover, when consumers have a greater amount of favorable feedback about a product or service, their inclination to make a purchase gets more intense, and the likelihood of making a purchase increase dramatically (Chang M.Y. et al., 2022). Consumer product engagement pertains to the level of interest and attention that consumers exhibit towards one particular food item (Liang Y., 2012).

Alternatively, we employ Zaichkowsky's definition, as referenced by Chang M.Y. (2022), to describe involvement in the following manner: "*The degree to which an individual views an object as significant, determined by their inherent needs, values, and interests."*

Rahman I. (2018) asserts that consumers develop engagement with a product when it possesses substantial or fundamental significance in their life. Furthermore, as stated by Beharrell and Denison, as referenced by Chang M.Y. (2022), engagement plays a crucial role in shaping one's ideas, attitudes, and behavioral consequences, such as the frequency of product consumption, through substantial cognitive effort.

In the same study, Chang M.Y. (2022) states that Strazzieri (1994) proposes that the level of involvement (high versus low) with the products under consideration is determined by the consumer's decision-making process. Hence, there exists a robust correlation between product knowledge and customer intention to purchase. The greater the level of buyer engagement, the more extensively they would utilize product facts.

"CLEAN LABEL" TERM FOR CONSUMERS

According to a 2015 consumer survey published by a publication of Wiley - Food Quality & Safety (2016), participants were asked to define the term "clean label." 34 percent of the respondents indicated that they were unfamiliar with the term "clean label." Respondents who claimed to understand the term "clean label" had differing opinions on the differentiating features of clean label foods. According to the Canadian survey mentioned before (2016), companies that are using clean labeling approaches may not be obtaining the necessary degree of consumer acceptance. According to a senior consumer insight analyst, the term "clean label" holds varying interpretations among consumers across different regions. Thus, the study revealed that a significant proportion of consumers, specifically 34 percent, lack any comprehension whatsoever of the concept. This elucidated also that the phrase is commonly employed in business to denote the increasing trend among consumers to prefer products that include "cleaner" attributes in terms of their component parts or production techniques.

For instance, eliminating high-fructose corn syrup or aspartame from products. The clean label

movement seems to be expanding as people demand the elimination of synthetic components from their food and shift towards consuming more natural food, also known as "clean eating."

According to the same survey conducted on the consumers, *"In the past few years, there has been a change in the way people approach diets. Instead of focusing solely on losing weight, there is now a greater emphasis on adopting a healthy and enjoyable lifestyle. Consequently, the term 'diet' has acquired negative connotations and is often contrasted with the clean eating movement."*

According to the Ingredion Clean Label Guide to Europe (2014), consumers in Europe are concerned about the contents of their food. This concern is reflected in their focus on the ingredient list, with 78% of customers finding it an important factor. Also, as mentioned in the same guide, in France and Italy, customers place the most priority on ingredients, with 86% and 89% respectively admitting that the selection of constituents is of great importance. Nevertheless, lesser documented levels of significance should not be dismissed. A potential reason why some consumers might give less importance could be attributed to the firmly established prevalence of the clean label movement in their country of origin.

These consumers usually have an expectation that their food will fulfill a specific clean label standard. This is especially accurate for markets such as the United Kingdom, where the clean label trend developed earlier and is nowadays more firmly developed. The clean label movement has gained momentum in countries like Turkey and Russia, with a rapid year-on-year growth. Therefore, these markets can be considered to be developing in a manner comparable to the market in the United Kingdom, where currently more than one-third of product releases are promoted as “clean label” and a “clean label” approach has become increasingly accepted as a must for achieving success on the market.

GENERATIONAL DIFFERENCES AND CLEAN LABEL PRODUCTS

In recent years, there has been a growing focus among food industry magazines and companies on the specific tastes of different generations of consumers when it comes to purchasing food products. Therefore, there is an increasing interest on consumer preferences and generational differences among Baby Boomers, Generation X, Millennials, and Generation Z.

Baby Boomers, born between 1946 and 1964, have assumed a subordinate role to their Millennial generation in several respects. Immediately after World War II, a significant number of baby boomers noticed the advent of ready-to-eat foods and the increased popularity of processed and packaged foods. For many years of their lives, the focus has been on owning many possessions and paying less attention to the transparency of their possessions. However, as individuals in this category age and experience the development of certain health problems, a significant number of them have shown an increasing awareness and concern for their well-being. Baby Boomers often choose clean-label foods primarily out of health concerns rather than a general preference for ingredient transparency. They opt for products that can help maintain their health, although they may not prioritize the complete avoidance of all artificial ingredients, unlike younger generations (FONA, 2018).

According to Mintel, as mentioned by FONA (2022), half of American customers aged 55 and above acknowledge that their motivation to eat properly stems from the desire to prevent disease or illness. The significance of preventive health measures is heightened by the fact that this generation is experiencing longer lifespans compared to previous generations. Consequently, individuals are adopting proactive strategies such as adhering to a nutritious diet and maintaining physical activity well into their senior years.

Boomers prioritize maintaining a healthy diet by focusing on their eating habits within their households. The proportion of individuals in this generation who link home-cooked meals with good health is notably more than that of their Gen Z counterparts (67% and 55%, respectively) (FONA, 2022).

Given that boomers prepare 80% of their meals at home and are over twice as inclined as Millennials to depend on leftovers, companies must prioritize the grocery store requirements of this generation (FONA, 2022).

According to the report Consumer Insight: Baby Boomers, published by FONA (2022), consumer purchasing patterns in the United States are as follows: 14% of individuals utilize online ordering for home grocery delivery, while 41% make use of digital coupons when shopping for groceries in physical locations. In terms of importance of value - Baby boomers exhibit brand loyalty when they sense distinct value and receive satisfactory customer service.

Regarding their purchases, the same report from 2022 claims that 49% of individuals express interest in functional foods such as probiotics and vitamins, while 59% are willing to pay higher prices for items that adhere to social compliance and sustainability standards. Products targeted toward the elderly may include a negative perception that

discourages individuals who do not identify themselves as being "old." Nevertheless, certain nutrients that promote brain health, eye health, and other benefits can be advantageous for individuals of all age groups (FONA, 2022).

Consequently, marketers can direct their marketing efforts toward baby boomers by emphasizing the same claims that attract younger consumers. Baby boomers, as a demographic, have strong preferences and tend to be resistant to trying new things. However, there are strategies to motivate these consumers to explore novel flavors or meals while still catering to their established preferences. An effective approach is incorporating novel ingredients into traditional recipes. According to the same report, 62% of Baby Boomers express a preference for experimenting with new foods if they are presented in a recognizable format on the menu (FONA, 2022).

Generation X, born between 1965 and 1980, have a unique point of view. They are the ones who witnessed the transition from an era of processed and ready-to-eat foods in their youth to the growth of the ecological movement and the clean label trend as they matured. They possess distinctive preferences, an appeal to nostalgia, a tendency toward skepticism, and exert a remarkable level of influence on nearly every subsequent generation. Also, having a genuine concern for their health and well-being, Gen Xers tend to be pragmatic in their choices. They value transparency and opt for products with a clean label, but occasionally they tend to choose less "clean" options, for the sake of nostalgia for the era of processed and ready-to-eat foods.

Marketers often disregard the Forgotten Generation, focusing instead on younger Millennials and elder Baby Boomers. According to Consumer Inside – Generation X Report (FONA, 2019), Gen X serves as a significant link between two extensively examined generations, and they possess an above-average household income and substantial purchasing influence.

As stated by Business Insider Report (2018), Generation X is the demographic group that is allocating the highest amount of money towards food in general. Instead, their emphasis lies on the quality of food, the capacity for customization, and attaining satisfaction. At the grocery store, convenience is the top priority for customers, and they are more receptive to novel and inventive concepts compared to past generations (FONA, 2019). According to Mintel cited in Consumer Inside – Generation X Report (2019), shoppers between the ages of 34 and 54 are significantly more inclined than shoppers aged 55 and above to express their willingness to consider meal planning and recipe suggestions as they buy. Generation X experiences a strong longing for the symbols and figures that were prominent during their early years of life. This is a method of establishing communication and fostering a connection with their Generation Z and Millennial children by demonstrating the significance of things that held importance to them throughout their own youth (FONA, 2019).

As stated by Food Business News cited by Consumer Inside – Generation X Report (FONA, 2019), Generation X depends on fruit snacks, corn chips, and granola bars, which are the same kind of food they relied on during their childhood. The desire for made-to-order or freshly prepared foods at supermarkets is mostly driven by Generation X. Mintel cited by Consumer Inside – Generation X Report (FONA, 2019), indicates that grab-and-go choices are also favorably regarded if they are promoted with an emphasis on freshness. Fried or rotisserie chicken is the most popular choice, indicating that Generation X prefers protein-rich main dishes rather than quick snacks. While it is preferable for the food to be nutritious, its nutritional composition is not yet a decisive factor (FONA, 2019). When making a purchase, the primary factor to consider is taste. Generation X has a strong affinity for comfort food, traditional cuisine, and characteristic dishes like pizza, and burgers are highly preferred. The most prevalent cuisines from other nations are Italian, Mexican, and Chinese (FONA, 2019).

Millennials, born between 1981 and 1996, have the potential for significant influence in the context of clean labeling. The current generation highly values authenticity, transparency, and sustainable products. The consumption patterns of millennials demonstrate a distinct emphasis on health and its impact on the environment. For this generation, selecting products that have a transparent and unambiguous label aligns with their principles and beliefs.

Consumers in this age group are inclined to conduct thorough investigations into food ingredients, demonstrate a willingness to pay higher prices for items that are clean products, and actively promote their health ideas and choices on social media, so exerting an influence on consumption trends.

Based on the 2022 Consumer Insight Report on Millennials published by FONA, millennials are currently in the advanced stages of their professional lives. Despite experiencing different challenges such as the COVID-19 pandemic and increasing inflation rates, millennials have encountered significant changes that have influenced their financial ability to spend.

Nevertheless, millennials desire their purchases to possess superior quality in terms of ingredients, flavor, and ethical considerations. When selecting items to purchase, 87% of individuals prioritize flavor as the most significant factor. While this age may exhibit a wide range of features, they unanimously prioritize taste as the paramount component in selecting a snack. According to Mintel cited in the same report published in 2022, 87% of millennials prioritize taste as the primary factor in their purchasing decisions (FONA, 2022).

Millennials, as a socially conscious demographic, possess a strong environmental awareness and have the expectation that their products align with this value. A significant majority of millennials, over 75%, are open to adjusting their purchasing patterns to acquire environmentally sustainable things. Furthermore, they are willing to pay additional costs to do so (FONA, 2022).

Nearly 40% of individuals belonging to the millennial generation have initiated a new relationship or reinforced an existing one with a company that has a favorable impact on the environment. According to statistics, a significant majority of millennials, over 90%, are inclined to support a company that they have confidence in when it comes to environmental matters. Furthermore, around 95% are likely to endorse and suggest that brand to their connections. In the 2022 report mentioned before, around one-third of millennials expressed their intention to allocate more of their budget towards the purchase of nutritious food and nonalcoholic beverages in the year 2023 (FONA, 2022).

When shopping for groceries, people prioritize healthier choices such as organic, fresh, low-fat, and low-sugar items. Overall organic food buying witnessed a 51% growth in 2019. A significant proportion of millennials, specifically 55%, prioritize convenience as a crucial determinant in their food purchasing decisions. The behaviors of millennials, who are constantly engaged in productive activities, are significantly influencing the perspective on dining occasions (FONA, 2022). According to a 2018 survey included in the mentioned report (FONA, 2022), 91% of millennials engage in snacking throughout the day to fulfill their energy and nutritional requirements. Additionally, 96% of them substitute a meal with a snack at least once a week, with lunch being the most common meal to be replaced. Additionally, they indulge in frequent snacking, with over half of them reporting eating 4-5 times a day. Despite enjoying frequent snacking, maintaining good health remains a primary concern. Nearly 90% of individuals have a "better-for-you" snack at least once a week. Among this group, protein is the key health feature desired in their snacks. Approximately half of the respondents consider protein to be the most crucial health aspect influencing their snack choices (FONA, 2022).

COVID-19 PANDEMIC IMPACTS ON FOOD SAFETY AND CLEAN-LABEL CONSUMER BEHAVIOR

The emergence of COVID-19 in the Wuhan region of China and its subsequent rapid spread to almost 50 countries worldwide has garnered significant global attention. This led to the World Health Organization (WHO) declaring it a pandemic on March 11, 2020 (Han S. et al., 2021). This virus exhibits a significant degree of similarity to two other coronaviruses that have appeared in the last twenty years like SARS-CoV, responsible for Severe Acute Respiratory Syndrome (SARS), and MERS-CoV, responsible for Middle East Respiratory Syndrome (MERS). Both viruses have caused significant rates of illness and death (Shereen M.A. et al., 2020). This virus is highly contagious through direct contact between individuals via respiratory droplets, which constitutes the primary mode of transmission. Additionally, contaminated objects, such as fomites, can also contribute significantly to the spread of the virus (Han S. et al., 2021).

Also, within a study published in 2012, Mullis L. et al (2012), shown that the virus can survive on food surfaces, making them potential carriers of the infection. The World Health Organization and the Center for Disease Control and Prevention (CDC) have stated that there is no evidence to suggest that SARS-CoV-2 can be transmitted or directly contaminate food and water (CDC, 2020).

However, it is important to consider the potential for the virus to spread through the consumption of food that has been in contact with contaminated surfaces, packaged in a contaminated environment, or handled and shared with an infected individual (Galanakis C.M., 2020). In this context, by Pung et al. (2020), stated there were cases of COVID-19 transmission during a conference in Singapore in January 2020. It was found that personal contact and sharing food were the means of transmission, indicating that food could potentially serve as a source of SARS-CoV-2 infection.

According to the Food and Agriculture Organization (FAO, 2020), COVID-19 impacted agriculture in two significant ways: by changing both the availability and the demand for food. These factors are closely tied to food security, which is consequently being jeopardized. The food supply

chain is a network that facilitates the movement of food from farms to consumers' tables through various stages such as manufacturing, packing, distribution, as well as storage (Chen S. et al., 2020).

Amidst the COVID-19 pandemic, the entire spectrum of the food supply chain, encompassing fresh veggies, fruits, bakery products, perishable foods, and food grains, have been severely damaged (Ivanov D et al., 2020). Thus, the COVID-19 pandemic has had a significant effect on food safety, which is one of the fundamental components of the food system (Galanakis C.M., 2020).

The COVID-19 pandemic has had a profound impact on consumer behavior, specifically concerning clean-label products since it has heightened focus on health, well-being, and food safety (Meixner O. et al., 2019).

Consumer interest in health and safety has risen, leading to substantial changes in purchasing patterns. There has been a clear movement towards more careful inspection of food ingredients and a continued demand for healthy options. Consumers generally increasingly link these choices with safety and health, particularly during the pandemic (Herrero M. et al., 2023).

Due to the restrictions implemented during the lockdown, there has been a rise in the demand for clean-label products. This is because customers are increasingly seeking healthier options during the crisis. Consequently, the understanding of what defines "clean" has progressed, encompassing more than just labels or products, but also encompassing elements of food safety and the ability of a product to remain fresh for an extended period, specifically concerning the transparency of the list of ingredients (Jiang Y. et al., 2021).

During the COVID and post-COVID period, there has been a predominant inclination towards products with positive nutritional claims and following clean label protocols. This is because there has been a significant increase in health-conscious purchases after the pandemic (Nicolosi A. et al., 2022).

In addition, despite the economic difficulties perceived during the public health crisis, consumers have exhibited a readiness to spend more money on clean-label food goods, indicating a preference for healthier, transparent, and perhaps safer choices (Van Bussel L. et al., 2022).

The pandemic has significantly impacted the global economy, thereby impacting food security, supply networks, and jobs. This has also resulted in an increase in digitalization and technology, along with its observed socio-environmental effects (Aday S. et al., 2020).

Owing to concerns regarding safety, the implementation of social distancing measures, and the adoption of lockdowns, there has been a significant transition towards online commerce. This is also related to the increasing demand for clean-label products since customers tend to seek precise and reliable food information while making online purchases (Gu S. et al., 2021). Automation of processes enhances productivity, quality control, and product traceability, all of which are crucial for upholding food safety standards. Therefore, clean-label products have earned the confidence of consumers by guaranteeing that they are manufactured in a safe manner and with total transparency (Rejeb A. et al., 2021).

Moreover, enhancing accuracy in manufacturing decreases food waste and guarantees the manufacture of safer food products for those who choose such options. Due to their distinctive characteristics, clean-label products concentrate on the use of simple, natural ingredients that are frequently regarded as recognizable and more secure by consumers (Saraiva A. et al., 2020).

Post-pandemic, customer expectations have shifted to prioritize the clarity, accuracy, and utility of food information. This reflects the increasing demand for clean-label products that offer transparent ingredient lists and origin information (Priya K. et al., 2023).

Due to the COVID-19 pandemic, there has been a noticeable surge in the demand for food safety. Consumer demand for food safety and quality has led to a rise in the popularity of clean-label products that emphasize natural ingredients. Given the ongoing increase in customer demand for natural, simpler, and safer products, the future of clean-label products appears optimistic in this context (Asioli D., 2017).

The findings of an online study including 346 participants reveal that consumers consider features such as minimal processing, removal of undesirable substances, and ethical considerations to be significant factors linked with clean labels. The consumer's perception of these traits includes the benefits of being healthy, socially responsible, appealing to the senses, having a reliable product, and being low in calories. Furthermore, canonical correlation analysis reveals two noteworthy connections between clean label features and their related advantages. The advantages of healthiness, low calories, and social responsibility are driven by the elimination of undesired components and the employment of familiar components. The qualities of being little processed and containing uncomplicated ingredients are linked to the advantage of enhanced sensory appeal (Cao Y. et al., 2023).

CONCLUSIONS AND FUTURE PERSPECTIVES

The shift of customers towards organic or clean-label products indicates a bright future for these food products, despite the challenges that producers encounter from several perspectives.

Producers in the food industry encounter challenges related to the manufacturing costs, creation, and marketing expenses of clean-label products. Furthermore, ensuring the procurement of raw materials that adhere to the quality criteria for such clean-label products is another crucial aspect. Furthermore, producers are required to overcome legal requirements to ensure the compliance of food items.

Consumers have a crucial role in ensuring the availability and longevity of clean-label items on the market. Therefore, they should actively endorse and promote transparent and clean products. Lastly, the brand associated with the label actively participates in the clean label campaign by aligning its identity and messages with the producer's.

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CLASSIFICATION AND CLINICAL SIGNIFICANCE OF PAPILLOMAVIRUS INFECTION IN DOMESTIC CATS

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Abstract

During the last decades, the infection with papillomavirus in domestic cats gained interest from the veterinary community due to its significant impact on the companion's animal's health. Therefore, in this review, we aim to present a concise classification of feline papillomaviruses and their clinical relevance in domestic felines. Initially, the different types of papillomaviruses affecting domestic cats are described. Here, we emphasize the molecular diversity and transmission ways to better understand each virus type and its clinical implications. Furthermore, we explore the clinical importance of papillomavirus infections, analyzing their various manifestations such as skin or oral lesions. We outline the signs and symptoms of these infections, shedding light on the oncogenic mechanisms used by the virus. The knowledge gained from this analysis holds the potential to refine veterinary medical practices, enabling the effective management of this condition and ultimately enhancing the overall quality of life for our feline companions.

Key words: Feline papillomavirus, molecular biology, skin and oral lessions, classification

INTRODUCTION

Papillomaviruses (PVs) are a group of circular DNA viruses with a double-stranded structure. Their genetic structure includes five or six early (E) genes and two late (L) genes. Typically, PVs are specific to certain species and exhibit a preference for specific types of epithelial tissues and even specific areas of the body (Doorbar et al., 2012). PVs are categorized based on the highly conserved L1 gene. If two PVs share 60–90% similarity in the L1 open reading frame (ORF), they are considered different types. PVs with less than 60% similarity are likely to belong to different genera (Bernard et al., 2010). Within a genus, these viruses often infect closely related host species, leading to similar lesions in those hosts (Bernard et al.,2010). Papillomaviruses have been discovered in a wide range of species, including mammals, birds and reptiles (Rector & Van Ranst, 2013). Most species are infected by multiple PV types, often from different genera (Bernard et al., 2010).

The papillomavirus life cycle is synchronized with cells' regular division and differentiation processes in the mucocutaneous stratified epithelium. Initial microtrauma provides the entry point for PV into basal cells. The expression of PV E1 and E2 genes enables the virus to generate a limited number of copies, which then infect neighbouring basal cells. Infection of these basal cells enables the persistence of PV, although viral replication only occurs when a basal cell differentiates terminally and transitions to the suprabasal layer of the epithelium. At this stage, the PV interferes with cell regulation through the action of E6 and E7 proteins, preventing terminal differentiation, retaining the nucleus, and compelling epithelial cells to divide and replicate the virus. As infected cells approach the epithelium's surface, the expression of L1 and L2 proteins facilitates virion assembly. Eventually, cells shed from the epithelial surface, and the natural degeneration of epithelial cells releases viral particles into the environment (Graham, 2017).

While it is well established that PVs are linked to various cancers, the progression from a PV infection to an associated cancer is a rare occurrence (Stanley, 2010). In terms of viral replication, the shift to cancer is typically a non-productive or 'dead-end' event for the virus. Persistent infection, which can last for several years in basal and stem epithelial cells, especially

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in the presence of at least one high-risk PV (HR-PV), seems to be the primary factor leading to cancer progression (Moody & Laimins, 2010). However, mere infection, even though necessary, is not adequate to fully trigger tumorigenesis. Regardless of the infected anatomical site, the crucial alteration required for advancing to malignancy involves heightened expression of the viral oncoproteins E6 and E7 in dividing infected cells. This increased activity of E6 and E7 can promote cell growth, impede differentiation, and induce chromosomal instability, ultimately leading to tumorigenesis. In the majority of cases, approximately 70% to 85%, alterations in oncoprotein expression occur due to the integration of the HR-PV genome into the host genome (Pett & Coleman, 2007).

Since PV infection does not lead to cell necrosis and its effects are primarily confined to the superficial layers of the epithelium, PVs typically trigger a mild inflammatory response. This phenomenon is particularly observed in slow-replicating and asymptomatic PVs (Doorbar, 2006). When the body mounts a response, it identifies and eliminates infected cells through a cell-mediated immune response (Egawa & Doorbar, 2017). This immune reaction can stop PV replication, and because PV proteins affecting cell growth are lost, any hyperplastic lesion induced by the infection tends to resolve. The onset of the cell-mediated immune response varies, explaining why some oral papillomas in dogs spontaneously vanish within three months while others persist for up to a year (Sancak et al., 2015). Despite lesion resolution caused by the immune response, PVs can persist in basal cells and likely continue replicating at a low rate. The immune system's role in controlling PV replication is evident in the lack of visible lesions resulting from the skin's ubiquitous infection with human betapapillomaviruses in immunocompetent individuals (Forslund et al., 1999).

In addition to causing the formation of hyperplastic papillomas, PVs have the capacity to disrupt normal cell regulation, potentially contributing to the development of cancer (Graham, 2017). In humans, PVs are the most common viral agents linked to cancer, particularly the high-risk alphapapillomaviruses, responsible for about 5% of all human cancers, including a significant portion of cervical squamous cell carcinomas (SCCs) and oral SCCs (Plummer et al., 2016). Similarly, in various domestic species such as horses, dogs, cattle, pigs, and sheep, PVs have been associated with neoplastic conditions (Borzacchiello & Roperto, 2008; Munday et al., 2020; Munday, Dunowska, et al., 2016; Sykora et al., 2017; Vitiello et al., 2017). However, it's crucial to note that the majority of PV infections in both humans and animals do not culminate in neoplasia. Other factors like the speed of the immune response or the presence of additional neoplasia-promoting factors play a pivotal role in determining whether a PV infection progresses to cancer (Doorbar, 2006; Munday, 2014).

MATERIAL AND METHOD

A comprehensive literature research was conducted using electronic databases such as PubMed, Scopus, and Web of Science. The search terms included "papillomavirus infections in cats," "feline papillomavirus classification," "types of lesions," and "neoplastic transformation in feline PV infections." Articles and studies published up to the date of the search were retrieved for analysis.

The retrieved literature was reviewed to understand the classification systems used for feline papillomaviruses. This involved analyzing molecular characteristics, genomic structures, and phylogenetic analyses of feline papillomaviruses. Special attention was given to the criteria and methods employed by various researchers and organizations in classifying these viruses into different types and genotypes.

Studies reporting clinical cases and pathological findings related to feline papillomavirus infections were reviewed. The focus was on identifying and categorizing the types of lesions associated with these infections. Lesions were classified based on their location, morphology, and severity. Detailed information on cutaneous, mucosal, and oral lesions was extracted and analyzed.

Special emphasis was placed on studies investigating the potential of feline papillomaviruses to induce neoplastic transformation. Cases reporting papillomavirus-associated cancers in cats were identified and thoroughly examined. Data on histopathological features, tumor types, and molecular markers indicating neoplastic transformation were collected and analyzed to understand the underlying mechanisms of papillomavirus-induced carcinogenesis.

CLASSIFICATION OF FELINE PAPILLOMAVIRUS

1. *Felis catus Papillomavirus* Type 1

The complete sequence of the first papillomavirus (PV) in domestic cats was published in 2002 (Tachezy et al., 2002). Initially named *Felis domesticus PV* (FdPV) 1, PVs identified in this species were later renamed *Felis catus PVs* (FcaPVs) to align with the correct taxonomic name for domestic cats, which is *Felis*

catus. Among FcaPVs, Felis catus PV type 1 (FcaPV1) is the sole known *lambdapapillomavirus* that infects domestic cats. Interestingly, FcaPV1 is closely related to *lambdapapillomaviruses* found in exotic felids (Rector & Van Ranst, 2013). Despite the discovery, there are limited reports of FcaPV1, leaving the age at which cats become infected and the proportion of infected cats unknown. Although FcaPV1 was first identified in a skin lesion, subsequent findings have confined its detection solely to the oral cavity (Munday et al., 2015; Munday & French, 2015).

2. *Felis catus Papillomavirus* Type 2

Among the papillomaviruses (PVs) that infect domestic cats, FcaPV2 seems to be the primary type behind diseases, garnering significant attention in feline PV research. Its full sequence was unveiled in 2009, classifying it as the sole member of the *dyothetapapillomavirus* genus (Lange et al., 2009). Cats typically contract FcaPV2 within the initial days of life, possibly during birth from the queen or through close contact while suckling and grooming (Thomson et al., 2015). FcaPV2 infection is widespread, with viral DNA detectable in skin swabs from nearly all clinically normal cats (Geisseler et al., 2016; Munday & Witham, 2010; Thomson et al., 2019). Furthermore, approximately a quarter of cats exhibit detectable serum antibodies against FcaPV2 (Geisseler et al., 2016). Intriguingly, FcaPV2 DNA and gene expression have been identified in blood samples from healthy cats, indicating viral replication in non-epithelial cells and the potential for viral transmission through blood or placenta (Altamura et al., 2018).

Studies have shown that *Felis catus* PV type 2 can disrupt normal cell regulation through various pathways. In human cancers, PVs contribute to cancer development by degrading the retinoblastoma protein (pRb), a crucial regulator in the cell replication pathway, thereby promoting cell division. Correspondingly, FcaPV2-infected lesions in cats exhibited intense p16 immunostaining, indicative of elevated p16 levels within cells (Munday et al., 2013; Munday et al., 2011a; Munday & Aberdein, 2012). Subsequent research confirmed that the FcaPV2 E7 protein binds to pRb, suggesting that FcaPV2 promotes cell division by E7-mediated degradation of pRb within cells (Altamura et al., 2016).

Additionally, 'high-risk' PVs in humans promote cancer by degrading the p53 protein. Although initial immunohistochemical (IHC) studies did not reveal a clear link between the presence of FcaPV2 DNA in lesions and the absence or presence of p53 protein (Munday et al.,2019), recent molecular studies demonstrated that the FcaPV2 E6 protein can promote p53 degradation within the cell, suggesting that this PV might interfere with normal p53 function (Altamura et al., 2016). Furthermore, FcaPV2 may enhance cell replication by upregulating mitogen-activated protein kinases and inhibit cell apoptosis by increasing protein kinase B expression (Altamura et al., 2016).

3. *Felis catus Papillomavirus* Type 3

The comprehensive genetic sequence of this specific papillomavirus type was initially documented in 2013 (Dunowska et al., 2014). FcaPV3's L1 open reading frame (ORF) sequence exhibits less than 60% similarity with both FcaPV1 and FcaPV2, but interestingly, it shows the highest resemblance to canine PVs in the *Taupapillomavirus* genus. This genetic and behavioral affinity led to the classification of this PV as the inaugural member of the species 3 *Taupapillomaviruses* (Van Doorslaer et al., 2018). Subsequent findings have identified FcaPV3 in hyperplastic and neoplastic skin as well as oral lesions in cats, indicating its capacity to influence cell regulation (Chu et al., 2020; Munday et al., 2018; Yamashita-Kawanishi et al., 2018). Moreover, lesions containing FcaPV3 DNA displayed prominent p16 immunostaining, suggesting the virus's ability to degrade pRb (Munday et al., 2018). The prevalence of asymptomatic FcaPV3 infections in cats and the precise timing of initial infections by this specific PV type remain unknown.

4. *Felis catus Papillomavirus* Type 4

The full genetic sequence of this particular papillomavirus (PV) was documented in 2014, following its discovery in a sample from a cat's mouth (Dunowska et al., 2014). Interestingly, the L1 open reading frame (ORF) of this virus exhibits the highest similarity to FcaPV3, leading to its classification as a species 3 *Taupapillomavirus* (Van Doorslaer et al., 2018). Rarely, this virus has also been found in cutaneous lesions in cats (Vascellari et al., 2019; Yamashita-Kawanishi et al., 2018; Yamashita-Kawanishi, Gushino, et al., 2021). Notably, in some cases where FcaPV4 was the sole PV type detected, it suggests that this PV has the potential to induce diseases in cats. Furthermore, the presence of FcaPV4 DNA has been linked to intense p16 immunostaining, indicating that this PV type might enhance cell replication by disrupting normal pRb function

(Munday, Gibson, et al., 2011). The prevalence of FcaPV4 infections in cats and the age at which cats are typically infected by this specific PV type remain unknown.

5. *Felis catus Papillomavirus* Type 5

This specific papillomavirus type was identified in 2017 from a skin lesion (Munday, Dittmer, et al., 2017). Although it has not been officially assigned to a genus yet, its genetic sequence closely resembles that of FcaPV3 and FcaPV4, indicating a probable classification as a species 3 *Taupapillomavirus*. Since its initial discovery, there have been occasional reports of FcaPV5 found in skin lesions in cats (Kok et al., 2019; Vascellari et al., 2019). The lesions linked to FcaPV5 exhibited p16 immunostaining, indicating that the virus might impact cell growth by disrupting normal pRb function (Munday, Marshall, et al., 2017).

6. Felis catus Papillomavirus Type 6

The latest papillomavirus type identified in domestic cats was fully sequenced from a skin lesion and was documented in 2020 (Carrai et al., 2020). Its L1 open reading frame (ORF) sequence shares the closest resemblance to FcaPV3, hinting at its probable classification as a species 3 *Taupapillomavirus*. Felis catus papillomavirus type 6 has not been reported subsequently, indicating that this specific PV type might be a rare culprit behind skin diseases in domestic cats (Munday et al., 2021).

7. *Bovine Papillomavirus* Type 14

While this specific papillomavirus type is capable of infecting cats, it is cattle that serve as the definitive hosts for BPV14. In cattle, this virus has been identified in papillomas (warts), bladder cancers, and normal skin samples (Da Silva et al., 2012; Munday & Knight, 2010; Roperto et al., 2016). In contrast, BPV14 has only been found in sarcoids, a type of mesenchymal neoplasia, in cats. Notably, this PV does not cause asymptomatic infections in cats, and cats appear to be dead-end hosts for this virus (Munday et al., 2010). Apart from domestic cats, BPV14 has also been detected in sarcoids from African lions and cougars (Munday, French, et al., 2011a).

FELINE PAPILLOMAVIRUS-ASSOCIATED LESIONS

1. Feline Viral Plaques and Bowenoid In Situ Carcinomas

Originally, feline viral plaques and Bowenoid in situ carcinomas (BISCs) were perceived as separate skin lesions. Nevertheless, given their common origin from PV infection and comparable histological characteristics, it is more accurate to regard them as varying degrees of severity within the same disease. To streamline discussions, this text will henceforth refer to all these conditions collectively as BISCs (Munday et al., 2021).

Bowenoid in situ carcinomas are rare skin lesions typically found in middle-aged to older cats. These lesions appear as multiple pigmented or non-pigmented, non-painful, non-pruritic, slightly raised growths, often up to 2 cm in diameter, commonly on the face, head, and neck (Wilhelm et al., 2006). Interestingly, these lesions can develop on both haired and non-haired skin, indicating that sunlight exposure might not be the primary cause. The expected behavior of BISCs remains poorly understood. Some smaller BISCs have been reported to regress spontaneously, but others persist and progress to invasive squamous cell carcinoma (SCC). While early reports suggested a link between immunosuppression and BISC development, many cats with BISCs do not have identifiable immunosuppressive diseases. Certain cat breeds, such as Sphinx and Devon Rex, are predisposed to BISCs, and these lesions tend to occur at an earlier age in these breeds. Moreover, in these specific breeds, BISCs progress more rapidly to invasive and metastatic SCC (Munday et al., 2016; Ravens et al., 2013).

Most BISCs have been linked to FcaPV2 infection. Studies using PCR primers designed to detect FcaPV2 found the virus in a significant number of BISC cases (Munday et al., 2007, 2008; Munday, French, et al., 2011b; Munday & Peters-Kennedy, 2010; Nespeca et al., 2006; Vascellari et al., 2019). In situ hybridization techniques have further localized FcaPV2 within the proliferating cells of BISCs (Demos et al., 2019; Vascellari et al., 2019). Although FcaPV2 appears to be the primary cause of BISCs, FcaPV3, FcaPV4, and FcaPV5 have also been associated with these lesions. Some regional differences in the predominant PV type have been suggested, and subtle differences in histological features caused by different PV types have been reported. For instance, FcaPV3 may cause proliferation of cells deeper within the hair follicle, while FcaPV5

infection might lead to proliferation of follicular structures as well as cells within sebaceous glands (Munday, Marshall et al., 2017). There is a hint, based on limited data, that BISCs caused by FcaPV3 might exhibit less aggressive behavior than those caused by FcaPV2 (Munday et al., 2016).

The mechanisms by which different FcaPV types induce hyperplasia and neoplasia remain unclear. However, intense p16 immunostaining has been observed in lesions containing FcaPV2, FcaPV3, FcaPV4, FcaPV5, and FcaPV6, indicating that all these PV types can disrupt normal pRb function (Carrai et al., 2020).

2. Feline Cutaneous Squamous Cell Carcinomas (SCCs)

Cutaneous squamous cell carcinomas (SCCs) are common in cats and are typically highly invasive, leading to significant morbidity and mortality. Similar to humans, most cutaneous SCCs in cats are believed to result primarily from sunlight exposure, occurring in non-haired, non-pigmented areas like the pinna, nasal planum, and eyelids. Although these SCCs often progress from actinic keratosis (a sun-induced intraepithelial neoplastic lesion) in sun-exposed skin, a smaller number develop in UV-protected areas, raising the possibility of progression from a Bowenoid in situ carcinoma (BISC) in these cases, although such progression has been documented in only a few instances (Munday, Benfell, et al., 2016; Ravens et al., 2013).

In 2006, DNA sequences from FcaPV2 were first identified in feline cutaneous SCCs (Nespeca et al., 2006). Subsequent studies showed more frequent detection of FcaPV2 DNA in cutaneous SCCs than in normal cat skin (Munday et al., 2008). Researchers from various parts of the world have also found PV DNA in feline cutaneous SCCs, with additional evidence supporting PVs' role in these cancers. Higher viral loads have been detected in BISCs and a subset of SCCs compared to normal skin (Thomson et al., 2016), and FcaPV2 gene expression has been identified and localized within feline cutaneous SCCs (Altamura et al., 2016; Hoggard et al., 2018; Thomson et al., 2016). Intense p16 immunostaining has been observed in SCCs containing PV DNA, highlighting the potential link between PV infection and cancer development, especially in cases with p16-positive SCCs showing longer survival rates (Munday et al., 2013; Munday, Gibson, et al., 2011).

Current evidence indicates that PVs influence around 30% of SCCs arising from UV-exposed skin and 75% of those from UV-protected skin (Munday et al., 2011). However, the precise impact of PV infection compared to other potential factors remains uncertain. FcaPV2 is the most commonly detected PV type in PV-associated cutaneous SCCs in cats, although a smaller proportion of SCCs contain FcaPV3, FcaPV4, or FcaPV6 DNA sequences (Carrai et al., 2020; Munday et al., 2011; Vascellari et al., 2019; Yamashita-Kawanishi et al., 2018; Yamashita-Kawanishi, et al., 2021). Regional differences have been observed, with FcaPV3 being most frequently detected in SCCs from cats in Japan (Yamashita-Kawanishi et al.,2021). Currently, whether the subsequent behavior of SCCs is influenced by the specific PV type causing the lesion remains unknown.

3. Feline Oral Squamous Cell Carcinomas (OSCCs)

Feline oral squamous cell carcinomas (OSCCs) are highly aggressive neoplasms that are almost always fatal. Currently, treatment options are limited, and cats diagnosed with these tumors typically survive for about 5 weeks on average (Klobukowska & Munday, 2016). While the cause of feline oral SCCs remains unknown, it is well established that a portion of human oral squamous cell carcinomas (OSCCs) are caused by papillomavirus (PV) infection (Schwartz et al., 1998). Given the role of PVs in human OSCCs and feline cutaneous SCCs, researchers have explored the possibility that PVs may also contribute to the development of feline OSCCs.

In the initial comprehensive study of feline OSCCs, PV sequences were detected in one out of 20 cancers but not in any of the 20 non-cancerous oral samples. Interestingly, the identified PV sequence in the feline OSCC belonged to a human PV type (Munday et al., 2009). In a subsequent study, PV DNA sequences were amplified from two out of 32 feline OSCCs. However, one of the sequences couldn't be sequenced, and the other was from a human PV type, raising concerns about potential sample contamination (O'Neill et al., 2011).

In contrast, no PV DNA was found in a series of 30 feline OSCCs from cats in New Zealand and in another study involving seven feline OSCCs from cats in Japan (Munday et al., 2011; Yamashita-Kawanishi et al., 2018). In a different New Zealand study, FcaPV1 sequences were detected in one out of 36 OSCCs and one out of 16 inflammatory gingival lesions, with no other PV types identified (Munday & French, 2015). A recent study in North America used advanced sequencing techniques to analyze 20 feline OSCCs

and nine samples of normal feline gingiva. Although various virus types were found, only one OSCC was found to contain a PV sequence, specifically from FcaPV3 (Chu et al., 2020). Conversely, an Italian study identified FcaPV2 in 10 out of 32 (31%) feline OSCCs and in four out of 11 (36%) samples of ulcerative gingivitis. The presence of gene expression in many positive samples indicated viral replication in oral tissues. However, efforts to confirm the location of FcaPV2 DNA within the samples through in situ hybridization yielded inconclusive results (Altamura et al., 2020). Additionally, FcaPV2 DNA sequences were found in 11 out of 19 (58%) OSCCs in a recent study of cats from Taiwan, although non-cancerous oral samples were not included in this analysis (Yamashita-Kawanishi, Chang, et al., 2021).

Several studies have reported varying levels of p16 immunostaining in feline oral SCCs. However, this variability has not been linked to the presence of PV DNA in feline OSCCs, suggesting that spontaneous mutations within the neoplasms might be responsible for the inconsistent immunostaining, rather than a PV-related cause (Altamura et al., 2020; Munday et al., 2019; Munday, Knight, et al., 2011; Yamashita-Kawanishi et al., 2018).

4. Feline Basal Cell Carcinomas (BCCs)

Basal cell carcinomas (BCCs) are much rarer in cats compared to squamous cell carcinomas (SCCs). Unlike SCCs, these tumors lack a connection to the overlying epidermis, and keratinization is generally absent. The neoplastic cells in BCCs are small and darkly basophilic, resembling cells found within the basilar layers of the epidermis (Goldschmidt et al., 2018).

The potential association between BCCs and papillomavirus (PV) infection in cats was first noted when it was observed that these lesions often occur alongside adjacent Bowenoid in situ carcinoma (BISC) lesions, and PV cytopathic changes were observed in cells within some BCCs (Gross et al., 2008). Subsequently, a cat with multiple BCCs containing FcaPV3 DNA was reported, indicating a link between PV infection and these tumors (Munday et al., 2018). Furthermore, short sequences from a novel PV type were amplified from a feline cutaneous BCC (Munday, French, et al., 2017). This PV type has not been fully sequenced, suggesting the likelihood of discovering additional PV types associated with cats in the future (Munday, French, et al., 2011a).

CONCLUSIONS

In conclusion, this article sheds light on the intricate classification and diverse lesions caused by feline papillomaviruses (FcaPVs). Through an exploration of various FcaPV types and their associated lesions, we have deepened our understanding of the complexities within this viral family. The identification and characterization of these lesions are crucial steps toward early detection, accurate diagnosis, and effective management of FcaPV-related diseases in domestic cats.

Furthermore, our analysis underscores the necessity for continuous research in this field. As new FcaPV types and their corresponding lesions emerge, it becomes imperative to stay updated and adapt diagnostic and therapeutic approaches accordingly. Collaborative efforts between researchers, veterinarians, and public health authorities are essential to enhance our knowledge of FcaPVs, leading to improved preventive measures and treatments.

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THERAPEUTIC ALTERNATIVES IN MAMMARY GLAND INFECTIONS IN COWS

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Abstract

Mastitis caused by infectious pathogens is still considered a ravaging disease of dairy cattle, affecting animal welfare. Economically, this condition appears in the dairy industry through reduced production performance and increased culling rates. Bovine mastitis is a mammary gland inflammation, most commonly caused by bacterial pathogens. Routine diagnosis is based on detecting clinical and subclinical forms of the disease. This highlights the importance of rapid detection of etiological agents at the farm level, for which several diagnostic techniques have been developed. Due to the predominance of bacterial etiology, treatment in mastitis is mainly based on the use of antibiotics. Nevertheless, antibiotic therapy has some limitations due to antimicrobial resistance, treatment efficacy, and costs at the farm level. Research needs to be directed toward developing new therapeutic agents/techniques that can replace conventional methods and address the problem of antibiotic resistance. The objective of the article is to briefly describe the current findings and results of herbal therapy as an adjuvant in the management of mammary gland infections.

Key words: bovine mastitis; treatment; herbs; therapy

INTRODUCTION

Mastitis is one of the bovine diseases, among the pathologies that particularly affect animal welfare and the economy. It adversely affects the profit benefits of livestock producers/farmers and leads to a large loss of production in the dairy sector worldwide (Bardhan, 2013; Sinha et al., 2014; Izquierdo et al., 2017; Aghamohammadi et al., 2018; Das et al., 2018). Mastitis in cattle is the mammary gland inflammation (intramammary inflammation, IMI) in cows. The disease is mainly caused by bacterial infections and is classified into two types based on epidemiology, namely contagious mastitis and environmental mastitis (Garcia, 2004). The former is caused by contagious bacteria, including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* spp. which are transmitted from an infected cow to a healthy cow, usually at the time of milking, via hands, towels, and the milking machine, serving as a reservoir for the bacteria. In contrast, environmental mastitis is caused by bacteria that are mainly spread outside the milking parlor, i.e. the causative bacteria originate from the cow's environment, such as bedding material, soil, manure, feces, and stale water (Garcia, 2004). Bovine mastitis leads to an increase in the cost of animal husbandry in terms of milk production. In addition, it also harms milk composition as well as milk value (Halasa et al., 2007; Kalinska et al., 2017). Environmental mastitis is strongly influenced by management practices (Garcia, 2004) and therefore requires better technical and biological tools along with appropriate incentives and encouragement. Farmers and field veterinarians need to work according to official guidelines for using approved antimicrobials (Klaas and Zadoks, 2018). Over the past century, significant progress has been made to keep mastitis under control; but due to changing population dynamics, herd structure, and more stringent processor standards that make mastitis is a complicated disease and remains a major problem in the dairy industry. Thus, further extensive research is called for (Ruegg, 2017a).

In cattle and buffalo, mastitis is an important economic problem worldwide, including in India (Das et al., 2018), Canada (Aghamohammadi et al., 2018), Germany (Hamann, 2001), the United

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Kingdom (Bennett et al., 1999), the Netherlands (Hogeveen et al., 2011) and the United States (Hadrich et al., 2018). Bovine mastitis is related to a daily loss that ranges from 1.0 to 2.5 kg of milk in the first two weeks after onset and a total loss of 110 to 552 kg throughout lactation, depending on parity and time of onset. Mastitis also has a long-lasting effect on milk production, as cows will not regain their maximum milk yield during the remaining lactation period (Rajala-Schultz et al., 1999). Despite various advanced dairy cattle and buffalo husbandry management practices, mastitis is still a threatening disease and is among the major economic problems of dairy farmers and dairy farm owners. India ranks first among the top milk-producing countries (cow and buffalo milk combined). Economic losses due to mastitis in India are about Rs. 575 million per year and reduce milk by 21% (Bardhan, 2013). In addition, consumption of milk that has been affected by mastitis can be harmful to humans because antimicrobial-resistant pathogens can be transmitted through contaminated unpasteurized milk; therefore, it is also a major public health concern/hazard (Oliver and Murinda, 2012). In addition, health risks associated with increasing microbial resistance and antibiotic residues in milk have led to increased consumer demand for organic products, as consumers consider food produced by conventional farming systems to be healthier and safer for consumption (Ruegg, 2009). Due to zoonotic threats, mastitis milk cannot be consumed and also cannot be sold; thus, contributing to major economic losses. Infected udder reduces the market price of animals and imposes an economic burden on the owner due to treatment costs (Gonzalez and Wilson, 2003; Seegers et al., 2003).

Although the association between mastitis and pathogenic microorganisms was established in 1887, the main pathogens were not identified until the 1940s. The discovery of the multifactorial etiology of bovine mastitis in the 1960s initiated further mastitis research (Singh and Singh, 1994; Ndlela et al., 2016), including the identification of common etiological agents, which are both Gram-positive and Gram-negative bacteria, such as *S. agalactiae*, *S. aureus*, *Escherichia coli* and *Klebsiella pneumoniae*; molecular epidemiology of causal pathogens; comparative pathogen typing methods at the subspecies level; virulence gene arrays; whole genome sequencing and *in vitro* antibiotic susceptibility pattern investigations. Over time, antibiotic therapy (penicillin) became available by 1945 but was not effective against all pathogens causing mastitis. There is a need for management practices targeting the pre-calving period in heifers to reduce the likelihood of mastitis in later stages (Naqvi et al., 2018). Generally, subclinical mastitis and IMIs in heifers during calving are predominantly caused by major pathogens, namely coagulase-negative staphylococci leading to mastitis in heifers. Early in the lactation period, IMIs are influenced by many factors, including the nature of the disease, the virulence of the pathogen, time of onset to calving, persistence of infection/cure, host immunity, gestation stage, and management practices, including risk associated with the season and herd location. Short-term antibiotic treatment before calving is an effective control measure for mastitis in heifers but is hardly ever recommended due to long-lasting adverse effects on udder health and milk production, thus reducing profitability for farmers (De Vliegher et al., 2012).

Mastitis diagnosis is an important demand of the dairy industry for safe milk production, not only for economic and public health reasons but also in terms of animal welfare. Diagnosis must be performed early, rapidly, and accurately for the prevention of mastitis or early detection of mastitis for management or therapeutic purposes. This involves the application of conventional as well as advanced diagnostic tests. Conventional methods are relatively cheap, easy, readily available, and applicable in the field, but usually non-specific. Advanced tests are expensive, and require technical skills and sophisticated infrastructure and facilities, but are usually accurate and specific for different forms of mastitis (Swarup et al., 1989; Singh et al., 2013; Hussein et al., 2018; Chakraborty et al., 2019).

Blanket dry cow therapy, strategic culling, and well-established biosecurity procedures are effective measures to control and prevent reintroduction of other virulent strains of *S. agalactiae* and *S. aureus* (Kefee, 2012). In addition, the combination of antibiotic treatment and culling of cows that do not respond to treatment has been shown to decrease transmission rates and reduce IMIs (Halasa, 2012). Several types of conventional and advanced therapeutic approaches are available for the management of mastitis, which includes antibiotics, vaccination, nanoparticle therapy, herbal therapy, and bacteriocins (Gomes and Henriques, 2016). Various agents help reduce udder infections, especially mastitis in cows and also help improve milk quality (Skowron et al., 2019). Out of these, antibiotic therapy and vaccination are commonly used methods for the treatment of mastitis. The extensive and uncontrolled use of antibiotics for treatment, along with the induction and persistence of biofilm-associated antibiotic resistance in

mastitis has caused a decreased response to antibiotic therapy (Park et al., 2012; Babra et al., 2013). Although vaccination is not effective against bovine mastitis because a variety of microorganisms are involved in its development, *S. aureus*, *Streptococcus uberis*, and *E. coli* have been considered to be the main targets for vaccine development (Wilson et al., 2009; Bradley et al., 2015; Collado et al., 2016; Ashraf and Imran, 2020). Although several commercial vaccines are available, most of them fail to provide sufficient protection and at the same time are expensive (Cote-Gravel and Malouin, 2019).

Mastitis-causing pathogens. The vast majority of pathogens causing clinical bovine mastitis are of environmental or ubiquitous origin. In comparison, contagious agents are mainly related to subclinical infections (Abebe et al., 2016; Klaas and Zadoks, 2018). Mastitis is a multi-etiological infection and some bacteria are mainly responsible for clinical, subclinical, contagious, and environmental mastitis. The most common bacteria involved are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Trueperella pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, and *Pasteurella* spp. (Levison et al., 2016; Abdalhamed et al., 2018; Shinozuka et al., 2018; Zhang et al., 2018). Out of these, the contagious pathogens are *S. aureus*, *S. dysgalactiae*, and *S. agalactiae*. *S. aureus* is the predominant organism, while the main environmental pathogens are members of the Enterobacteriaceae, notably *E. coli* and *S. uberis* (Petersson-Wolfe et al., 2010). *S. agalactiae* is the most common Gram-positive bacteria in clinical mastitis, followed by *S. aureus*, while *Klebsiella* spp. and *E. coli* were the most isolated Gram-negative bacteria in clinical mastitis (Cortinhas et al., 2016). *S. agalactiae* and *S. aureus* disseminate mainly through contact, so herd biosecurity can be considered an important preventive measure to minimize and eliminate the reservoirs (Kefee, 2012).

Both clinical and subclinical forms of mastitis can be attributed to most bacterial pathogens. However, *T. pyogenes* is exclusively responsible for causing clinical forms of mastitis (Malinowski et al., 2006). In primiparous cows, the highest milk loss is due to *S. aureus*, *Klebsiella* spp., and *E. coli*. In older cows, substantial losses are due to infections with *Streptococcus* spp., *T. pyogenes*, *S. aureus*, *Klebsiella* spp., and *E. coli* (Grohn et al., 2004). In general, *S. aureus*, *S. agalactiae*, and *S. uberis* are common pathogens causing mastitis, while *Mycoplasma bovis* and *Corynebacterium bovis* are less often involved (Wernicki et al., 2014; Vakkamaki et al., 2017). Coagulase-negative staphylococci and their role in causing mastitis should also be seriously considered (Krukowski et al., 2001). Wilson et al. (1997) reported that *S. agalactiae* and various pathogens, including *Prototheca* spp., *Streptococcus* spp., and *T. pyogenes*, are associated with most cases of mastitis. Mastitis shows its most severe form when it is associated with infections due to coliforms, CAMP-negative *Streptococcus* spp., *T. pyogenes*, *S. agalactiae*, fungi (yeast-like), and *Prototheca* spp. (Wilson et al., 1997; Malinowski et al., 2006). *Corynebacterium* spp. (40%) and *S. aureus* (32%) were the most frequent isolates found by Steele and McDougall (2014) in subclinical mastitis cases in New Zealand. *Prototheca* spp. are pathogenic algae and opportunistic pathogens that cause mastitis in dairy herds and pose zoonotic potential (Alves et al., 2017; Dos Anjos et al., 2019).

S. aureus was the most common pathogen identified in cases of mastitis (McParland et al., 2019). Methicillin-resistant *S. aureus* (MRSA) CC22-MRSA-IV was reported as an intramammary pathogen by Magro et al. (2018). On genotyping by DNA microarrays, MRSA was noted as an epidemic UK-EMRSA 15 grouping in CC22. These isolated strains had resistance genes for β-lactams and macrolides. The isolates were obtained from milkers and dairy cows, suggesting reverse zoonosis. Routine milk sampling and evaluation identified the presence of mastitis-causing pathogens in 13% of all samples obtained from dairy herds. Out of the pathogens isolated, *S. aureus*, *Streptococcus* spp., *T. pyogenes*, and *C. bovis* were found to be the most common pathogens (Cvetnic et al., 2016).

Type of mastitis and clinical relevance. Epidemiologically, mastitis can be classified into contagious and environmental mastitis and is caused by a broad spectrum of pathogens. Furthermore, mastitis can also be categorized as clinical or subclinical forms (Garcia, 2004; Abebe et al., 2016). Any increase in humidity and pollution in the barn environment also increases the load of bacterial pathogens in the environment. One study showed a 74.7% prevalence of mastitis in the herd and 62.6% in cows. Regarding subclinical and clinical mastitis, the former type seems to be responsible for the majority of cases (59.2%) compared to the latter (3.4%) (Abebe et al., 2016). Clinical mastitis can be easily identified based on obvious symptoms in terms of inflammation of the udder, which shows redness in the affected part or the whole udder, increased temperature, swelling, pain on touch, milk clots, discoloration, and changes in milk consistency. General symptoms are pyrexia (> 39.5 C) and loss

of appetite. Environmental pathogens, including coliforms, induce major causes of clinical mastitis. Of the 20,000 cases of clinical mastitis in the Netherlands, 40% were caused by *S. uberis* and *S. dysgalactiae*, 30% by *S. aureus*, and 30% by *E. coli* (Steeneveld et al., 2011). Cow udder can show decreased susceptibility as well as resistance to the inflammatory state under certain conditions. These conditions include the administration of antibiotics to the udder for prolonged periods, a higher incidence of udder mycosis due to mineral-vitamin and antioxidant deficiencies, dietary imbalance, poor environmental conditions, and climate change (Wawron et al., 2010).

Kumar et al. (2010) have studied the incidence and economics of clinical mastitis. Compared to the clinical form, in subclinical mastitis, there are no clinically visible symptoms, although a change in milk composition may be an indicator. It is therefore recognized and confirmed by laboratory examination of milk or by animal tests such as the California mastitis test (CMT) followed by laboratory isolation of the aetiological agent.

The somatic cell count (SCC) in milk should be less than 200,000 per ml in a healthy cow. Somatic cells are mostly white blood cells (WBC), i.e. infiltrating neutrophils, as well as macrophages into mammary gland tissue as a result of inflammation (Akers and Nickerson, 2011). *S. agalactiae* localizes mainly in the udder and causes persistent infections with higher SCC (Kefee, 2012).

Mastitis is the result of the host's immune response to infectious agents affecting the udder (Gurjar et al., 2012). Usually, there is a balanced microflora in a healthy udder. The intramammary microbiota is made up of a complex community of various bacteria (Rainard, 2017; Andrews et al., 2019). The commensal mammary microbiota present in the healthy udder plays a major role in immune homeostasis (Derakhshani et al., 2018). As a result, a disruption of the diversity of the microbiota in the udder (dysbiosis) can have an important effect on the udder. The normal microbiome of the udder is an important factor to consider when making a diagnosis of mastitis, as healthy quarters also contain some bacteria. Different bacterial genera such as *Ruminococcus, Oscillospira, Roseburia, Dorea, Prevotella, Bacteroides, Paludibacter,* and *Bifidobacterium* are usually present in the udder. Any injury or congenital anomaly of the udder or teat, such as teat fistula, leaky teat, and udder injury that exposes the udder to external microbes or milk retention tends to cause mastitis (Rambabu et al., 2011).

In one study, tissue affected as a result of mastitis showed significant inflammation with marked decreases in alveolar epithelium and lumen, while histopathologically, an increase in stromal connective tissue was reported along with leukocytosis (Nickerson et al., 1995). These types of conditions either expose the udder to external pathogens or weaken internal resistance. In the clinical form of mastitis, *Staphylococcus* spp. or *E. coli* predominates and the normal microbiota is disrupted. The researchers proposed that because of either alteration of the normal microbiome due to pathogens or prolonged use of antibiotic therapy, mastitis is induced and established (Falentin et al., 2016). In a detailed molecular epidemiological study, most dairy cattle in the U.S. were found to carry more than 10 species of coagulase-negative staphylococci (CNS), and the bacteria were isolated at various stages of lactation (Jenkins et al., 2019).

Mastitis is a complex of negative outcomes of various factors acting together at the host level. These factors include pathogens, their growth pattern in the udder parenchyma, signaling pathways for the establishment of clinical manifestations, and various molecular mechanisms mediated by pathogen-associated molecular patterns (PAMPs). Doing so is possible through various host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-like receptors (RIGs), which trigger inflammation of the udder due to microbial infections, along with several environmental factors. Therefore, it requires a collaborative approach to diagnosis as well as control of this important disease (Bhattarai et al., 2018).

Advances in treatment of mastitis. An effective and efficient mastitis control program involves early identification of infection through understanding the pathogenesis, development of new sensitive tests for early detection, adoption of good management practices to reduce the possibility of transmission, and prevention of uninfected transmission. The control program should include the strategic use of antimicrobials to reduce problems with antibiotic residues in milk and antimicrobial resistance (Ruegg et al., 2017a). Before drug therapy, the primary cause of udder infection should be clarified. Teat or udder conditions such as teat fistula, leaky teat, teat spider, and udder wounds require immediate attention. Because such conditions rupture the effective barrier and tend to expose the teat canal or udder to external microbes; therefore, prompt remedy is required.

Successful treatment of clinical mastitis depends on several factors: antimicrobial treatment, identification of the causative agent, parity, lactation status, history of previous SCC, clinical mastitis, and other systemic disorders (Steeneveld et al., 2011).

In a study conducted under Dutch circumstances, the cow-specific treatment recommended for clinical mastitis was not found to be economically beneficial (Steeneveld et al., 2011). Still, herd-specific interventions, such as cow-specific treatment and culling strategies against subclinical and clinical IMIs, can turn out to be highly cost-effective in managing mastitis (Gussmann et al., 2019).

Management of mastitis involves both preventive and therapeutic strategies and is mainly based on antibiotic therapy. However, recent approaches used to treat mastitis involve the usage of natural therapies, such as zeolites and propolis, which could potentially act as an alternative to antibiotic therapy (Benic et al., 2018).

Herbal therapy. Herbal therapy is a promising area in the treatment of mastitis as there are no associated adverse effects. Ethno-veterinary medicine is a branch of veterinary medicine that focuses on the treatment of diseases with herbal formulations (Tiwari et al., 2018). Herbs can be used as a therapeutic alternative or as an adjuvant in the management of mastitis in cattle. They can be used as an antibacterial, anti-inflammatory, and immunomodulatory agent for the treatment of mastitis (Mushtaq et al., 2018). The anti-inflammatory and antibacterial effects of Chinese herbs have been effectively used in the treatment of bovine mastitis (Muluye et al., 2014; Yang et al., 2019). Also, they can be used as a substitute for antibiotics and antipyretic drugs that are commonly used in the treatment of mastitis (Muluye et al., 2014). Ranjith et al. (2018) reported that methanolic extracts of herbal preparation that contain *Diploclisia glaucescens* leaves and *Curcuma longa* rhizomes in equal ratios produced analgesic activity along with anti-inflammatory activity. The analgesic activity of the herbal extract was found to be comparable to that of ibuprofen and indomethacin (Ranjith et al., 2018). Herbal therapy involves a variety of routes of administration depending on the type of formulation. Of these, topical routes (Hase et al., 2013), oral administration (Dash et al., 2016), and intramammary routes (Yang et al., 2019) are the most commonly used methods. In a comparative study performed to evaluate the efficacy of homeopathic complex therapy, herbal therapy (Neem seed extract), and antibiotic therapy for the treatment of subclinical mastitis in dairy buffaloes, antibiotic therapy was found to have superior efficacy compared to herbal therapy (Neem seed extract) and homeopathic complex therapy groups. When the cost factor was taken into account, herbal therapy was found to be the least expensive (Younus et al., 2018). Therefore, it can be effectively used as an adjuvant to antibiotics in the treatment of clinical mastitis without causing a large change in the cost factor. Some herbal extracts may have anti-inflammatory and antioxidant values that help heal udder inflammation and minimize oxidative stress. *Moringa* extract has been found to ameliorate inflammatory mediators and enhance antioxidant systems in bovine udder epithelial cells. This inhibited the expression of proinflammatory cytokines (TNF-α, IL-β, and IL-6), cyclooxygenase-2 expression and reduced NF-kβ, increased heme-oxygenase-1, NAD(P)H and quinone oxidoreductase-1, besides that *Moringa* extract increased the expression of casein proteins (Cheng et al., 2019).

Several plant species are used for the prevention and control of bovine mastitis in southern Brazil due to their anti-inflammatory, immunomodulatory, and antibiotic effects (Avancini et al., 2008; Xu et al., 2015). The leaves, bark, bulbs, and aerial parts have been used to prepare medicinal plants. The leaves, bark, bulbs, and aerial parts have been used to prepare medicinal plants. Species of plants such as *Achillea millefolium, Allium sativum, Alternanthera brasiliana, Baccharis trimera, Chenopodium ambrosioides, Cuphea carthagenensis, Foeniculum vulgare, Phytolacca dioica, Sambucus nigra, Sida rhombifolia, Solanum mauritianum, Atractylodis macrocephalae Koidz,* and *Solidago chilensis*, have been used orally, of which *Alternanthera brasiliana*, *Baccharis trimera* and *Sambucus nigra* have also been used as topical agents. *Ocimum basilicum* and *Parapiptadenia rigida* are the two plant species that have been used intramammary in bovine mastitis (Avancini et al., 2008). *Staphylococcus epidermidis* is one of the major causes of medical device infections and bovine mastitis due to its biofilm-forming ability. *Oxytropis glabra* is a Fabaceae species that is extensively used as a Chinese herbal formula in western China. *In vitro* studies performed to evaluate the effect of *O. glabra* decoction on *S. epidermidis* biofilm formation have identified a potential inhibitory mechanism that can be further explored in the development of new drugs against biofilm-associated infections (Ren et al., 2020). In one study evaluating the efficacy of *Ocimum sanctum* leaf juice as supportive therapy for the management of chronic staphylococcal-induced

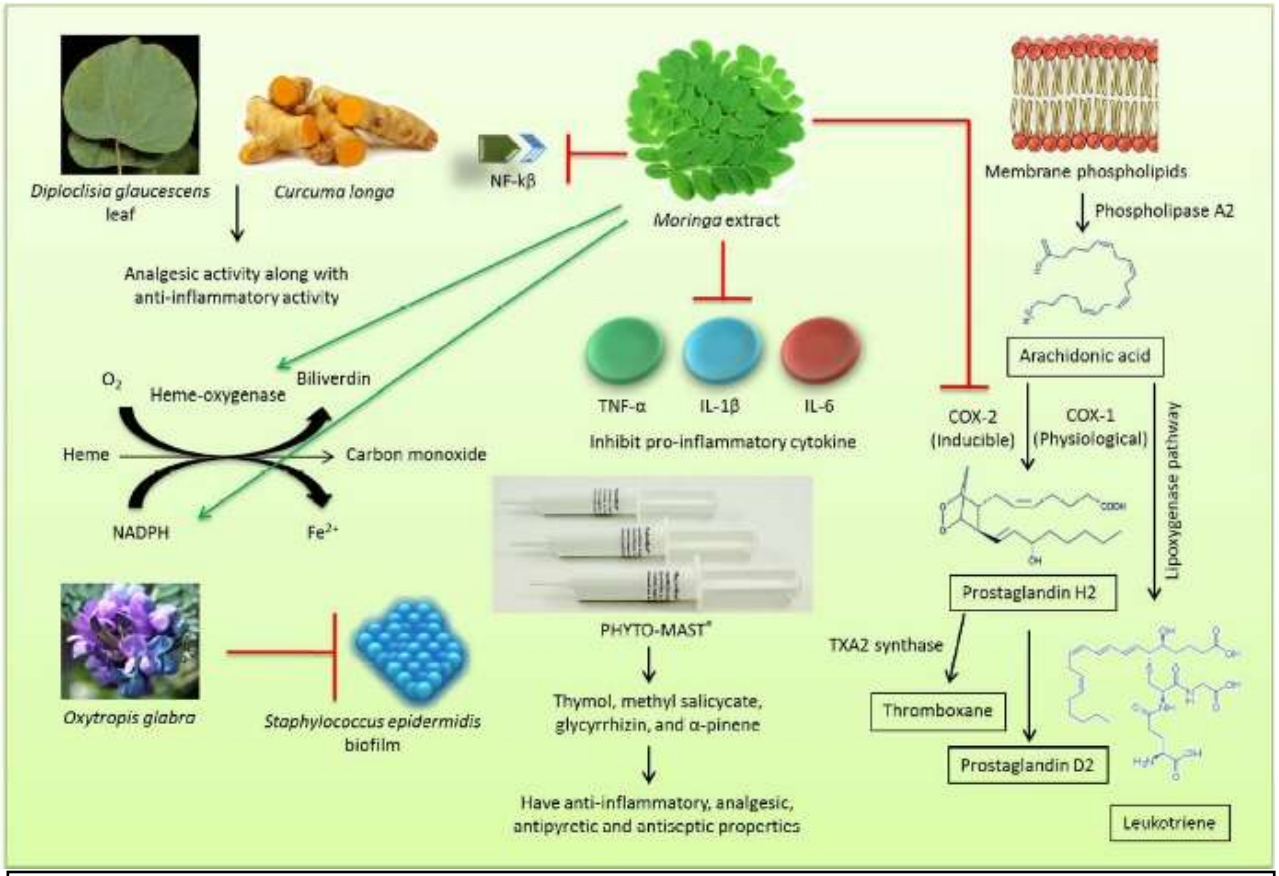

Figure 1. **Herbal therapy for the treatment of mastitis. Various herbs possess anti-inflammatory and analgesic properties**

mastitis, it was found that the leaf extract had significant bio-enhancing and antioxidant activities, which can be effectively used in combination with antibiotics (Dash et al., 2016). Therefore, instead of using herbal therapy as a single agent in the management of clinical mastitis, better results can be achieved if they are included in the treatment protocol as an adjuvant, along with other modalities of therapy. In a recent study aimed at evaluating the *in vitro* antibacterial activity of ethyl acetate extract of the *Terminalia chebula* plant against molecularly identified isolates of *S. aureus, E. coli, Pseudomonas aeruginosa,* and *Bacillus megaterium*, it was observed that a concentration of 500 μg/mL of extract had the same antibacterial efficacy as standard amoxicillin (Kher et al., 2019). This result provides insight into the potential of plant extracts to replace antibiotics as a single agent in the management of clinical mastitis.

Botanical preparations, such as PHYTO-MAST®, have ingredients (thymol, methyl salicylate, glycyrrhizin, and α-pinene) considered within the „Generally Recognized as Safe" United States Food and Drug Administration (FDA). The ingredients have anti-inflammatory, analgesic, antipyretic, and antiseptic properties and may be effective in treating mastitis (McPhee et al., 2011). However, one study failed to demonstrate any therapeutic effect after 3 days of repeated intramammary application at 12-hour intervals (Pinedo et al., 2013).

An overview of the role and mode of action of herbal therapy for the treatment of mastitis is shown in Figure 1.

CONCLUSIONS

Mastitis affects animal well-being and causes economic and production losses through deterioration of milk quality, reduced production performance, increased culling rate, cost of treatment, and due to mortality associated with the acute form of the disease. Several strains of microorganisms can cause both clinical and subclinical forms of the disease. Subclinical mastitis is more economically important than clinical mastitis because of its ability to deteriorate the quality of milk to such a high level that it cannot be detected at first sight, but will affect the overall quality.

Once mastitis is diagnosed, the main challenge for the veterinarian or producer is to manage the animals in such a way that they do not get more severe and become an economic loss to the production system. Several therapeutic strategies, such as antibiotics, vaccines,

bacteriocins, herbal therapy, immunotherapy, and nanoparticle technology, were evaluated for their effectiveness in treating mastitis, but no single technique is effective in controlling or treating the disease because of the variable response of the etiological agents to therapeutic techniques. To date, antibiotics have been widely used as the main therapeutic agent in the management of mastitis, but with the emergence of bacterial resistance, which has arisen due to the uncontrolled use of antibiotics, several other treatment options are being investigated. The development of a universal therapeutic agent/technique that can be seen as a substitute for antibiotic therapy is a necessity of this century.

One such therapeutic agent/technique can overcome the emerging problem of bacterial resistance. Future research should be directed towards advanced therapeutic strategies that can provide a solution to the current situation.

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HAEMATOLOGICAL CHANGES IN CANINE PARVOVIRUS INFECTION

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Abstract

Canine parvovirus (CPV) infection is a highly contagious disease caused by canine parvovirus type 2 and commonly produce acute gastrointestinal illness. All dogs are susceptible to CPV, although some dogs are at greater risk than others, as puppies (between 6 weeks of age and 6 months), unvaccinated or incompletely vaccinated dogs. Due to the virus multiplication and effect on the bone marrow, severe haematological changes are reported, resulted also from the combination of severe inflammation, gastrointestinal bleeding and depletion of hematopoietic cell lines. In the present study, haematological changes in dogs diagnosed with CPV infection were analysed.

Keywords: dog, CPV, haematological changes.

INTRODUCTION

Canine parvovirus (CPV) is a highly contagious infectious disease produced by an DNA virus from family *Parvoviridae*, genus *Protoparvovirus.* Important pathogens in this genus include feline panleukopenia virus (FPV) and closely related mink and raccoon parvoviruses, which have existed for over 100 years, and canine parvovirus (CPV), which arose as a variant in the mid-1970s and in 1978 spread worldwide, causing a disease pandemic among dogs, wolves and coyotes (ICTV).

CPV commonly causes gastrointestinal disease in young and unvaccinated dogs. Young (6 weeks to 6 months old), unvaccinated or incompletely vaccinated dogs are considered to be the most susceptible (ICTV).

CPV preferentially infects and destroys rapidly dividing cells of the small-intestinal crypt epithelium, lymphopoietic tissue and bone marrow. Destruction of the intestinal crypt epithelium results in epithelial necrosis, villous atrophy, impaired absorptive capacity, and disrupted gut barrier function, with the potential for bacterial translocation and bacteraemia (MSD Veterinary Manual). Viral replication occurs initially in the lymphoid tissue of the oropharynx, with systemic illness resulting for subsequent haematogenous dissemination. In the present study, haematological changes in dogs diagnosed with CPV were analysed.

MATERIALS

In the present study were included 10 dogs (6 males and 4 females) diagnosed with CPV infection, at the Infectious Diseases Clinic, Faculty of Veterinary Medicine, January to October 2023. The age ranged between 4 and 8 months, mixed breed and without any history of vaccination. A complete history of the dogs included: the age, breed, sex and origins, as well the clinical examination (general body condition, weight, respiratory and heart rate, mucous membrane color and body temperature, capillary refilling time).

The most commonly observed clinical symptoms were represented by lethargy, loss of appetite, bloody diarrhea, vomiting and dehydration. In order to established the CPV diagnosis, rectal swabs were collected and tested

for the presence of antigen, for both CPV and canine corona virus (CCV), using a rapid quantitative immunoenzymatic test, on V-Check equipment. The coefficient of infection (COI) ranged between 2.6 and 57. As high the COI is, the more concentrated in virus a sample is considered to be. Besides rapid testing, cell blood count (CBC) analysis was performed, by venipuncture of the cephalic or jugular vein, for each dog. Haematological analysis was performed using the haematological analyser to determine the RBC, Total Leukocyte Count (TLC) and the Pack Cell Volume (PCV).

RESULTS

All the rapid tests were positive for CPV infection and none for CCV infection.

The haematological analysis values were different on each case. The most frequent imbalance was noticed at white blood cells ranging from 0 to 20.74 10^9/L (range 6-17 10^9/L) observed in 6 dogs, thrombocytes 10-123 10^9/L (range 165-500 10^9/L) observed in 6 dogs, red blood cells 2.23-8.59 10^12/L (range 5.5-8.5 10^9/L) in 5 dogs, haemoglobin 3.5- 21.1 g/dl (range 12-18 g/dl) observed in 5 dogs, haematocrit 11.5-60.98% (range 37-55%) in 5 dogs, lymphocyte 0.25-6.2 10^9/L (range 1-4.8 10^9/L) in 4 dogs and neutrophils 13.56-15.48 10^9/L (range 3-12 10^9/L) in 4 dogs.

After the therapeutic protocol (5-7 days) all the patient's recovered successfully.

No agent-specific treatment has proven effective, therefore treatment remains symptomatic and supportive. Even no specific drug is available against CPV infections, heterologous hyper immune immunoglobulins known as „CANGLOB P" were used. These immunoglobulins ensure passive immunization of dogs against CPV. Specific antibodies facilitate to prevent the development of disease or to alleviate its course. On intravenous administration, an immediate onset of the passive immunity is recorded and utilization of immunoglobulins is the highest. After intramuscular and subcutaneous administration, a slightly delayed onset of the passive immunity is recorded, with this immunity being lower as compared with the intravenous administration. The dose is of **0.4 ml / kg body weight (**irrespective of breed, age and sex), for 3-5 days.

The protocol implemented, included also intravenous fluid therapy, symptomatic and hygienic-dietary treatment. The main goals include restoration of fluid, electrolyte, and metabolic abnormalities and prevention of secondary bacterial infection. Correcting dehydration, replacing ongoing fluid losses and providing maintenance fluid needs are essential for effective treatment. Dogs must be monitored for development of hypokalaemia and hypoglycaemia. If electrolytes and serum blood glucose concentration cannot be routinely monitored, empirical supplementation of IV fluids with potassium and dextrose is appropriate.

Antibiotics are indicated because of the risk of bacterial translocation across the disrupted intestinal epithelium and the likelihood of concurrent neutropenia. A beta-lactam antibiotic will provide appropriate gram-positive and anaerobic coverage. Second- or third-generation cephalosporin's can also be considered for their relatively wide spectrum of activity against Gram-positive and negative bacteria. Antibiotic therapy is typically only needed for 5–7 days.

Antiemetic therapy as maropitant (1 mg/kg, IV or SC, every 24 hours) and ondansetron (0.5 mg/kg, slow IV, once; then 0.5 mg/kg, IV infusion, for 1 hour) appear to be equally effective at controlling vomiting. Metoclopramide (0.2–0.5 mg/kg, IM or SC, every 6–8 hours) may be considered as an antiemetic as well as for its prokinetic effects, particularly in dogs with significant gastric stasis. Other drugs, such as anti-haemorrhagic, analgesics, antacids and gastric mucosal protection and others, are used in the complex treatment of CPV.

DISCUSSIONS

Anemia, leukopenia and thrombocytopenia are commonly reported, due to the virus effect on the bone marrow, resulted from the combination of severe inflammation, gastrointestinal bleeding and depletion of hematopoietic cell lines (Urbani et al., 2022).

Most dogs develop a moderate to severe leukopenia characterized by lymphopenia and neutropenia. Leukopenia, lymphopenia and the absence of a band neutrophil response within 24 hours of starting treatment has been associated with a poor prognosis (MSD Veterinary Manual). Leukopenia is due to destruction of hematopoietic progenitor cells in the bone marrow, depletion of lymphoid tissues and consumption of peripheral neutrophils due to massive demand in the inflamed gastrointestinal tract (Corda et al., 2023). Thrombocytosis, pancytopenia, neutrophilic leukocytosis and monocytosis may also occur (Mylonakis et al., 2016).

In the study of Castro et al. (2013) on 50 puppies diagnosed with CPV infection, leukopenia, lymphopenia, thrombocytopenia, hypoglycaemia and

hyperproteinaemia were all correlated with this viral infectious disease. Leukopenia and hypoglycaemia were related to poor survival in CPV-infected puppies. Leukopenia, lymphopenia and thrombocytopenia were frequent among dogs infected with CPV compared with the control group.

In the study of Khare et al. (2020), the frequent haematological abnormalities observed in CPV was anaemia. Anaemia, but also leukopenia may be because the virus affects bone marrow and is cytotoxic for hematopoietic cell leading to myeloid and erythroid hypoplasia during acute stage of the disease. The haematological changes are widely accepted to be attributable to destruction of hematopoietic progenitor cells of the various leukocyte types in the bone marrow and other lymphoproliferative organs such as the thymus, lymph node and spleen.

In the study of Bhargavi et al. (2017), the haemato-biochemical alterations noticed in CPV affected dogs were represented by anemia (57.14%) leucopenia (42.86%), neutropenia (28.57%), lymphopenia (42.86%), thrombocytopenia (35.71%), elevated BUN (50%), hyperproteinemia (21.43%), hypoglycemia (21.43%), hypokalemia (35.71%) and hypochloremia (42.86 %).

In the study of Terzungwe (2018), the distribution by sex revealed that only male dog had lymphocytosis, while both female and male dogs had neutropenia. Both vaccinated and unvaccinated dogs shared neutropenia and monocytosis, with moderate lymphocytosis in the unvaccinated dogs. Neutropenia, monocytosis and lymphocytosis are among the consistent findings. Neutropenia and monocytosis were observed in dogs younger than 6 months, while lymphocytosis was observed in dogs younger than 6 months. There was a significant difference in red blood cell count and packed cell volume among age groups.

CONCLUSIONS

Leukopenia due to lymphopenia is the most consistent haemotological finding associated with CPV infection in dogs. Severe neutropenia often occurs due to virus induced myeloid degeneration of the bone marrow and the extensive loss of neutrophils through the damaged intestinal wall.

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TAILORED MANAGEMENT PLAN FOR PREVENTING VIRAL DISEASES WITHIN DOG SHELTERS

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Abstract

Dog shelters play a crucial role in animal welfare by providing temporary care and finding permanent homes for homeless dogs. However, the close confinement and high turnover of animals in shelters create an ideal environment for the spread of viral diseases. To combat this, various preventive measures have been implemented, but a comprehensive and tailored approach is essential to effectively safeguard the health of shelter dogs. This study outlines a tailor-fit management plan that incorporates a multifaceted approach, encompassing vaccination, testing, hygienic practices, and quarantine protocols, to effectively prevent viral diseases within dog shelters.

Key words: viral disease; dog shelters; tailor-fit management; immune-prophylaxis

Introduction

Dog shelters play a crucial role in providing temporary care for homeless dogs and facilitating their adoption into loving homes. However, these environments often pose a significant risk for the spread of viral diseases among the shelter population. Viral diseases can cause significant morbidity and mortality in dogs, leading to reduced welfare and limiting the shelter's ability to effectively place animals. Therefore, implementing effective preventive measures is essential to safeguard the health and welfare of shelter dogs and minimize the risk of disease outbreaks.

Previous studies have shown that many dogs entering a shelter will have an insufficient antibody titer against viral but preventable diseases. Restricting vaccination to some dogs, and excluding others based on source, health status, false negative result or any other criterion contributes to the risk of transmission of infectious diseases in the shelter (Velescu, 2002; Lechner et al., 2010; Monteiro et al., 2016). Among the methods of preventing viral diseases in a shelter are vaccination, hygiene, avoiding overpopulation, and reducing the period of stay of animals in the shelter.

The occurrence of viral infections at a shelter may undeniably have a catastrophic impact on the health and well-being and may pose significant challenges even for shelters that are well-prepared and well-equipped. While a definitive solution to eradicate illnesses like parvovirus or Carré disease has not been found, significant progress has been made in treatment alternatives since their inception. (Perley et al., 2020). Many dog shelter organizations have begun to adopt various treatment strategies in their efforts to save more lives (Appel and Barr, 2009). Due to the significant losses caused by viruses, especially parvovirus, and the high costs of treatment of sick animals, 3 important problems arise:

- ✓ How can limited resources (infrastructure, personnel, medical supplies) be used without compromising the health and welfare of shelter animals?
- ✓ What preventive measures are more effective in case of viral diseases?
- ✓ Can a personalized plan for viral disease management be developed within each shelter?

TAILORED STRATEGY BASED ON SPECIFIC NEEDS

A comprehensive approach to disease prevention in dog shelters requires a tailored strategy that addresses the specific needs and characteristics of the shelter population and environment. This involves considering factors such as:

Shelter Size and Capacity: Shelter size and capacity influence the potential for disease spread and the feasibility of implementing certain prevention measures. Larger shelters may require more robust vaccination programs and enhanced biosecurity protocols.

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Shelter Location and Socioeconomic Factors: Shelters located in areas with higher disease prevalence or socioeconomic challenges may need to prioritize vaccination and disease surveillance efforts.

Shelter Population Demographics: The age, breed, and health status of the shelter population influence the risk of particular viral diseases. For instance, young puppies and unvaccinated dogs are more susceptible to parvovirus and distemper.

Vaccination Programs: Vaccination is the cornerstone of disease prevention in dog shelters. A comprehensive vaccination program should include core vaccines, such as those against parvovirus, distemper, adenovirus, and rabies. Shelters should also consider vaccinating against other relevant diseases based on the local prevalence and shelter population characteristics.

Biosecurity Practices: Biosecurity measures aim to prevent the introduction and spread of infectious agents within the shelter environment. These measures include:

Strict Entry and Exit Screening: Implementing clear protocols for entering and exiting the shelter premises, including thorough cleaning and disinfection of boots and clothing, can help prevent the introduction of pathogens from outside sources.

Separation of New Arrivals and Established Groups: Newly admitted dogs should be kept separate from the established shelter population for a period to allow for observation for signs of illness and potential infectious diseases.

Strict Cleaning and Disinfection: Regular cleaning and disinfection of kennels, common areas, and equipment are essential to minimize the accumulation of pathogens.

Personal Hygiene: Shelter staff and volunteers should maintain strict personal hygiene practices, including frequent handwashing and appropriate use of personal protective equipment.

Disease Surveillance and Outbreak Management: Prompt identification and management of disease outbreaks are crucial to prevent widespread transmission and protect the health of the shelter population. This involves:

Regular Monitoring: Shelters should implement regular health screenings and observation of dogs for signs of illness, with particular attention to vaccinated dogs that may still be susceptible to certain diseases.

Prompt Diagnosis and Reporting: Upon detection of clinical signs suggestive of viral diseases, shelters should promptly seek veterinary diagnosis and report the incident to local authorities.

Isolation and Quarantine: Suspected or confirmed cases of viral disease should be isolated from the general shelter population to prevent further transmission.

Disinfection and Environmental Cleaning: After an outbreak, thorough cleaning and disinfection of the affected areas are essential to eliminate pathogens and prevent recurrence.

TAILOR-FIT MANAGEMENT PLAN FOR PREVENTING VIRAL DISEASES WITHIN DOG SHELTERS

Assessment of resources available to implement surveillance, disease control and treatment measures

The first step in creating a viral disease management plan appropriate to each shelter is to assess the resources available to implement measures to monitor and combat the disease and treatment. The purpose of this evaluation is to determine which animals can be successfully treated without endangering the health and welfare of those or other animals in the shelter (McCaw and Hosking, 2006; Appel and Barr, 2009). Given the highly infectious and deadly nature of parvovirus, having the physical ability to prevent transmission of the virus to other animals is of utmost importance. Vaccination is the cornerstone of parvovirus prevention in shelters and communities. In the absence of maternal protection, a single modified live vaccine can confer protection in 3-5 days. Research conducted to date has found that currently available vaccines protect against all known strains of parvovirus, including CPV-2c (Kelman et al., 2020). All dogs and puppies > 4 weeks of age should be vaccinated at the time of admission to the shelter (or ideally at least one week before), including those that are injured. Revaccinating puppies every two weeks until they are 18 weeks old, as long as they remain in the shelter, and revaccinating adult dogs at least once, 2 weeks after the first vaccine or after adoption may result in a decrease in incidence. Parvovirus spreads mainly through feces, also through vomiting and other bodily excretions. Dogs can become infected through direct contact, contaminated objects (food and water containers, leashes) and even aerosolization during surface cleaning (Lamm and Rezabek, 2008; Behdenna et al., 2019; Khatri et al., 2017; Van Arkel et al., 2019). At the shelter level, transmission of the virus can be fostered by several factors (Figure 1).

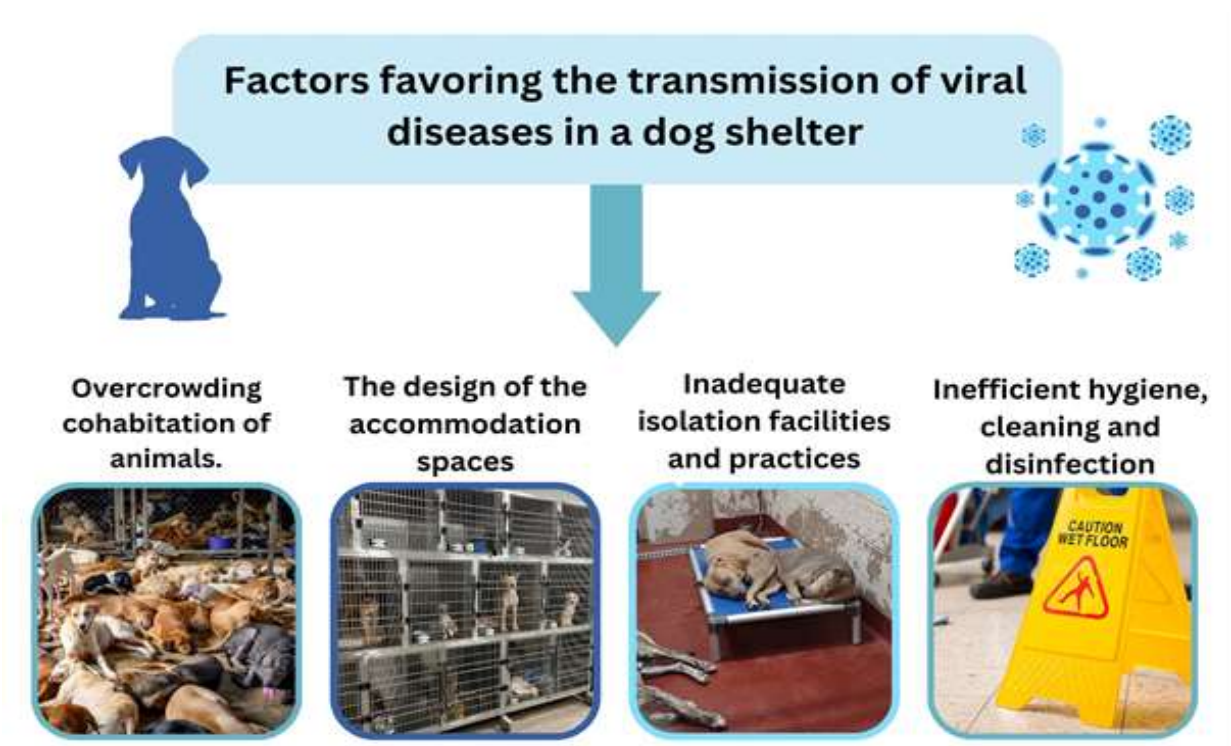

Figure 1. Factors favoring the transmission of viral diseases in dog shelters

The incubation period is usually 4-6 days, but the duration of incubation may be affected by the viral load to which the animal is exposed. It is recommended that animals be quarantined for at least two weeks after known exposure (Velescu, 2002; Goddard and Leisewitz, 2010; Perianu et al., 2012). Viremia usually occurs 2-3 days before the appearance of clinical signs (day 3-7), as early as day 1 after exposure (Figure 2).

In a crowded shelter where early signs might be missed, the viremia period may go unnoticed. Thus, this aspect must be taken into account when assessing the risk of dogs exposed to infection. Elimination of the virus can continue up to 2 weeks after recovery of the animal. A negative parvo ELISA in a dog that was initially diagnosed positive suggests that the virus is no longer eliminated in significant amounts.

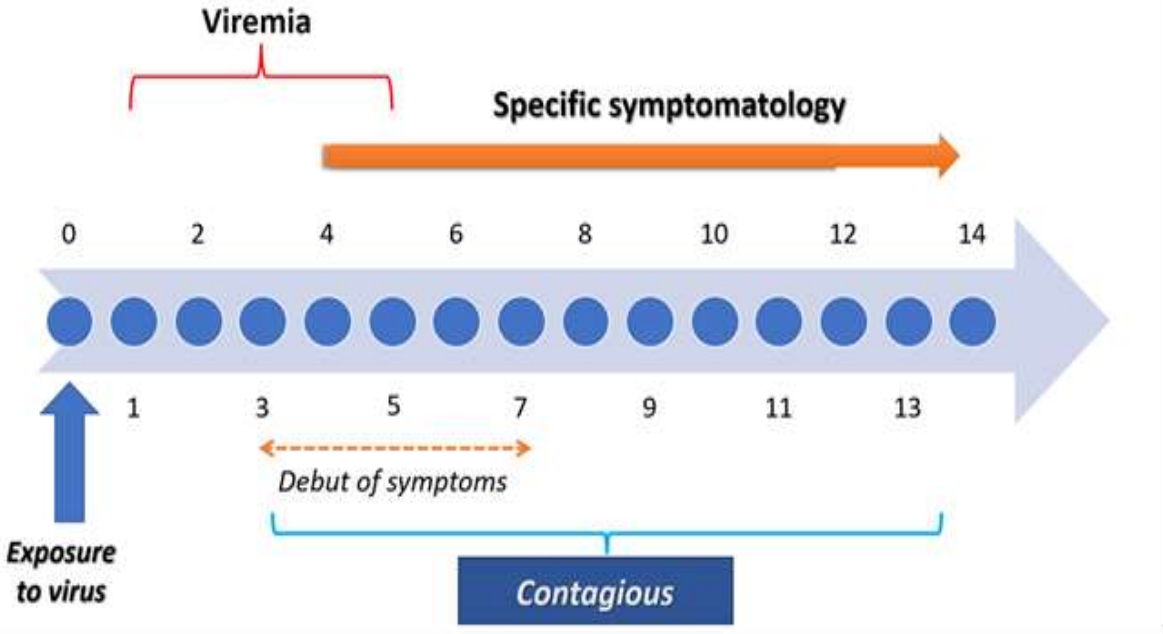

Figure 2. Parvovirus – duration of incubation period, viremia and specific symptoms

The virus can remain viable for months and years, especially in a dark and humid environment, so hygiene and disinfection using proven virulicidal biocidal products is essential. Effective options include 5% sodium hypochlorite (household bleach) freshly mixed with water in a ratio of 1:32 and applied to a clean surface. Products from the same family as bleach that have proven effective include calcium hypochlorite and sodium dichloroisocyanurate. Like bleach, they have no detergent properties and must be applied to a pre-cleaned surface. Other proven products include potassium peroxymonosulfate and accelerated hydrogen peroxide, which both have higher detergent properties and better activity against organic matter compared to bleach and related products (Howie et al., 2008; Eterpi et al., 2009).

Independent studies have repeatedly shown that quaternary ammonium disinfectants do not reliably kill parvovirus, despite repeated reformulation and claims of efficacy on the label. Pens, cages and accommodation spaces must be thoroughly cleaned, disinfected and dried at least twice before reuse. For areas such as courtyards and alleys where disinfection is not an option, careful and repeated mechanical cleaning can be effective if applied correctly (Sanekata et al., 2010; Addie et al., 2015; Cavalli et al., 2018).

In order to prevent the spread of diseases, it is essential to use appropriate protective equipment when interacting with sick animals (gloves, disposable overalls, booties for shoes). When treatment is carried out on-site, it is preferable to achieve it by using a free-standing, bio-safe isolation unit. In addition to adequate infrastructure facilities, human resource is especially important, since the management of this pathology requires adequate medical expertise. Veterinary input is essential, but it is particularly important that all staff and/or volunteers are properly trained, as prevention, diagnosis and treatment of viral diseases often require a significant investment of time to ensure adequate monitoring of patients and in some cases even the administration of treatments several times a day.

Finally, analyzing the shelter's budget is important to ensure that medical treatment is also financially feasible. The average duration of treatment for parvovirus is usually at least one week, so sufficient resources should be available at the shelter level to support a treatment plan for that length of time (Gerlach et al., 2020; Horecka et al., 2020).

In addition to assessing physical, staff, temporal and financial resources, an individual animal welfare assessment should be made. One of the fundamental goals in veterinary practice is that "treatment should not be worse than disease." This is a very real possibility in severe cases of parvovirus, therefore, the decision whether or not to treat these cases should also consider the benefit of treatment for that animal. Conducting treatment for animals that have a good prognosis and setting objective parameters of unacceptable welfare conditions to define treatment endpoints will help ensure success and adherence to personalized treatment protocol within each shelter. Treatment of parvovirus must consider a number of essential components such as avoiding and correcting dehydration, preventing secondary bacterial infections and treating endoparasites, ensuring proper nutrition and relieving discomfort to enhance well-being and accelerate recovery (Velescu, 2002; Perianu et al., 2012). Managing each of these components is essential for a positive outcome and all should be given equal importance. Hygiene and good biosecurity practices are also essential components of an effective parvovirus management plan at the shelter level. Taking into account resources and well-being, parvovirus treatment can be a successful and rewarding experience. An effective treatment protocol should result in recovery of 60% to 90% of patients.

Strategies to prevent viral diseases at dog shelter level

Viral diseases such as parvovirus or Carré disease are highly contagious and are a significant problem in dog shelters. That is why it is important for shelters to develop protocols to help prevent the introduction of the virus and effectively manage disease if it enters the shelter population. An effective viral disease management program takes into account both the animal and the space where it is housed, as well as the personnel with whom the animal comes into contact. Preventing exposure is one of the fundamental measures to avoid the introduction of the virus into the herd, but in a shelter, this is extremely difficult to achieve. Strengthening the immune system of dogs in the shelter by ensuring proper conditions and balanced nutrition can help increase the immunity of dogs and thus decrease their susceptibility to viral agents.

Reducing stress and carrying out appropriate vaccination schedules also helps prevent infections in the herd. Co-infection with gastrointestinal parasites is common, especially in puppies infected with parvovirus. Such infections can worsen the severity of the infection and delay recovery. Because the specific treatment protocol for each parasite species varies, administration of a

broad-spectrum dewormer such as ivermectin, fenbendazole may be used when a definitive diagnosis of coproparasitic infection is not possible or conclusive.

Hygiene and cleanliness within the shelter is crucial to prevent the occurrence and spread of viral diseases. Both spaces where animals and common objects are kept should be sanitized periodically. It is also important that protective equipment for single use is used when handling sick animals and in areas intended for quarantine and treatment of animals.

Given the contagiousness of viral diseases and the resources needed to treat them, shelters must properly assess their capacity for optimal care, ensuring they have sufficient supplies, medicines, and staff to meet the animals' physical needs and medical performance in the best conditions (Figure 3).

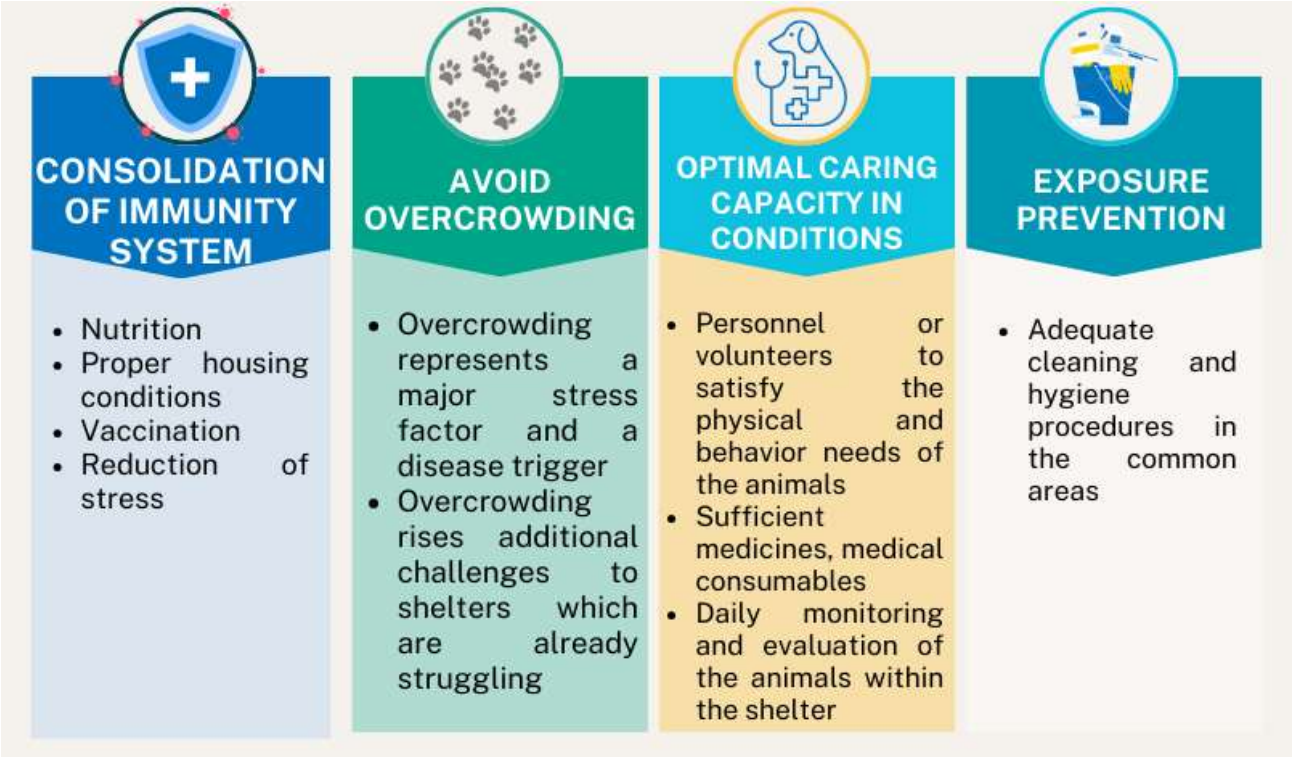

Figure 3. Strategies to prevent viral diseases in dog shelters

Conclusion

By implementing a tailored management plan that combines vaccination programs, biosecurity measures, disease surveillance, and outbreak management, dog shelters can effectively prevent the spread of viral diseases and safeguard the health and welfare of the shelter population. This approach not only contributes to the welfare of individual dogs but also ensures that shelters can continue fulfilling their mission of providing temporary care and facilitating adoptions.

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EFFECTS OF LUMBAR INTERVERTEBRAL DISC HERNIATION ON ADJACENT MUSCULATURE ON COMPUTED TOMOGRAPHY (CT) EXAMINATION

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Abstract

In the late 1800s Dexler used for the first time the term IVDD (Intervertebral disc disease), which was described as the presence of cartilaginous material in vertebral canal. Intervertebral disc herniation (IVD) refers to the part of the disc that is within vertebral canal.
The aim of the paper was to highlight the morphometric changes of the dorso-lumbar musculature occurring in dogs diagnosed with the presence of hyperattenuating material in vertebral canal by CT.
Thirteen dogs from varying breeds of dogs (French bulldog, Dachshund, Bichon, Pekingese) age from 2 to 8 years old, with hind limbs locomotory disease were scan with a Somatom Scope 16-slices CT scan. **Results**: Pronounced muscle contracture was seen on CT scan thus: 1,4 mm $^{(+-0,6mm)}$ in Bichon on left multifidus muscle in the area of the protrusion, 1.6 mm $^{(+-0,6mm)}$ in Dachshund on left multifidus muscle in the area of the protrusion, 2,1 mm $^{(+-3\ mm)}$ in Pekingese on left multifidus muscle in the area of the protrusion and 1,2 mm $^{(+-0,5\ mm)}$ in French bulldogs on the right multifidus muscle in the area of the protrusion.
An increased muscle contracture was seen in all patients in the area of the herniation.

Key words: herniation, ct scan, IVD

Introduction: In the late 1800s Dexler used for the first time the term IVDD (Intervertebral disc disease), which was described as the presence of cartilaginous material in vertebral canal. Intervertebral disc herniation (IVD) refers to the part of the disc that is within vertebral canal (Fenn J et al, 2020). In almost all the cases the IVD herniation can occur after a physical activity such as jumping or running and just in few cases can occur spontaneously (Tamura S et al, 2015).

An emerging area of research in veterinary medicine is the analysis of epaxial muscle size and composition in connection to intervertebral disc disease (IVDD) and lumbosacral stenosis (Bostrom A et al, 2022). The epaxial muscles in dogs with spinal disorders show atrophy comparable to those seen in humans (Bostrom A et al, 2022). Dogs with progressive lumbosacral stenosis were found to have multifidus muscle atrophy and muscle stiffness and function loss are caused by changes in muscle structure (Tokunaga A, and Shimizu M, 2020).

Conservative therapies (rest and the prescription of anti-inflammatory and analgesic drugs) and surgical decompression are available for dogs with intervertebral disc disease (Steffen F et al, 2014).

Surgical decompression is the standard treatment for non-ambulatory dogs. Over 90% of dogs with incomplete injuries recover independent ambulation and continence with surgical care. Nearly 60% of dogs with functionally complete injuries eventually recover, but the prognosis is less favorable (Zidan N et al, 2018).

The aim of this study was to highlight the morphometric changes of the dorso-lumbar musculature occurring in dogs diagnosed with the presence of hyperattenuating material in vertebral canal by CT.

MATERIAL AND METHOD

Thirteen dogs were part of the current study. Seven males, aged 2-8 years and six

females, aged 3-6 years, of the breeds Bichon, Dachshund, Pekingese and French Bulldog.

All patients were seen in the Veterinary Radiology and Imaging Clinic of the Faculty of Veterinary Medicine in Cluj-Napoca, coming from different backgrounds. All patients were diagnosed with various locomotor disorders.

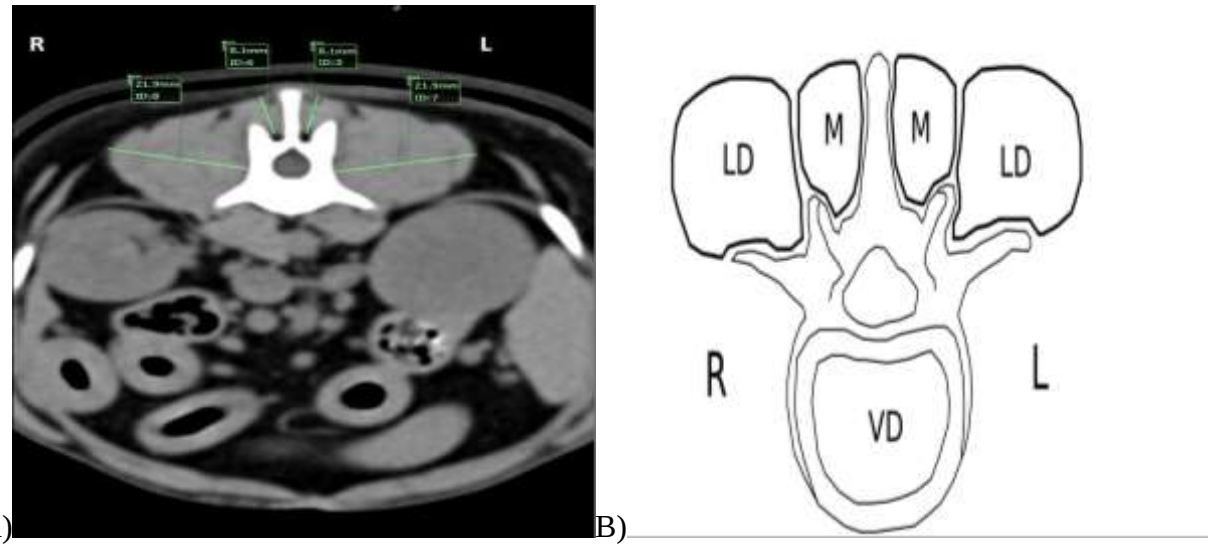
All patients were scan with a Somatom Scope 16-slices CT scan. All images were acquired in the local PACS and the measurements were done through an oblique line on the muscle, in Biotronics 3D Dicom Viewer.

Normal values for dogs without any tomographic spinal changes (at first three lumbar vertebras) are shown in table 1 and the methodology for measuring in the figure 1. M1 comes from Multifidi muscles and M2 comes from Longissimus dorsi muscle. The measured values are shown in figure 2.

RESULTS AND DISCUSSIONS

	L1 mm				L2 mm				L3 mm			
Muscle	M1R	M2R	M1L	M2L	M1R	M2R	M1L	M2L	M1AR	M2R	M1L	M2L
Means	7.1	21.9	7.2	22.1	8.1	21.9	8.1	21.9	8.2	22.2	8.2	22.3
SD	2.1	1.4	1.8	2.6	1.2	1.4	2.2	3.2	3.1	2.5	1.32	1.7

Table 1. Normal values



A) B)

Figure 1 A) CT scan at level of L2 – original; B) Schematic draw of the vertebral body and adjacent musculature (M-Multifidi muscle, LD- Longissimus dorsi muscle and VD – vertebral body); Source: Bostrom A et al, 2022)

In Bichon patients we obtain the following results: in the area of disc protrusion on the left side a 0.6 mm more pronounced contracture is observed compared to the left side. The dorsolumbar musculature covering the vertebra anterior to the disc protrusion shows a 1.4 mm more pronounced contracture on the left side and the dorsolumbar muscle covering the vertebra posterior to the disc protrusion shows a 2.9 mm more pronounced contraction on the left side. on the right side.

In the Dachshund breed in the area of disc protrusion on the left side, a more pronounced contraction of 1.6 mm is observed on the left side. The superficial dorsolumbar muscle, anterior to the site of protrusion, shows a 2.2 mm more pronounced contracture on the right side, while the deep dorsolumbar muscle shows a 2.3 mm more pronounced contracture on the right side. The dorsolumbar muscle covering the posterior vertebra at the site of protrusion is 1.5 mm more contracted on the right side

In the Pekingese breed - in the case of right-sided disc protrusion a 2.4 mm more pronounced muscle contraction is observed on the right side compared to the muscles on the left side. The dorsolumbar muscles of the vertebra anterior to the

disc protrusion site show a 5.0 mm more pronounced contraction on the right side. The dorsolumbar muscles of the vertebrae located posterior to the disc protrusion site show a 2.1 mm more pronounced contracture on the left side than on the right side.

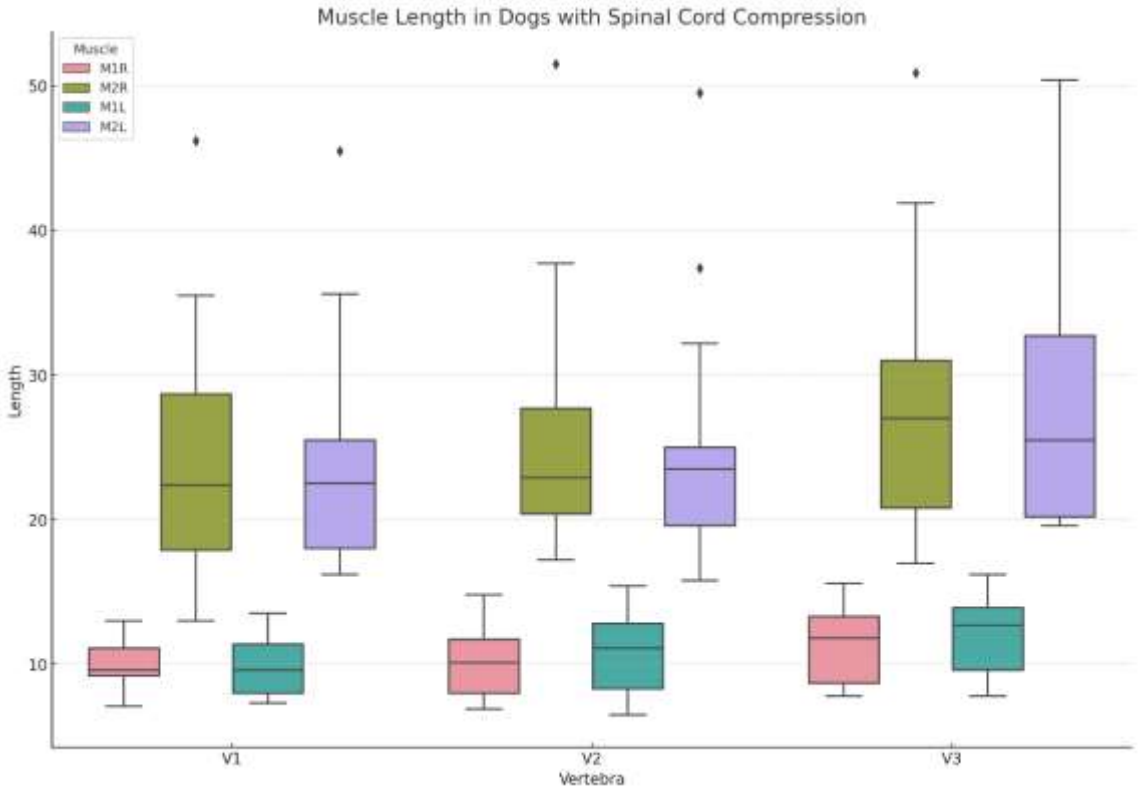


Fig. 2 Value distribution in dogs with spinal cord compression

In the French Bulldog breed in the case of right-sided ventro-dorsal disc protrusion, a 1.5 mm more pronounced muscle contraction is observed on the left side at the protrusion site than on the right side. The muscles on the vertebra anterior to the disc protrusion show a more pronounced contraction on the left side by 1.2 mm. The muscle on the posterior vertebra of the disc protrusion is 0.6 mm more contracted on the right side.

For diagnosing changes that occurs at the spine level, after a neurological examination, radiography can be the next step but is a screening tool method with some limitations, while CT and MRI are more accurate for diagnosing diseases from the spine and vertebral canal (Jeffery NC, et al, 2013; da Costa et al, 2020).

In both dogs and people, IVD degeneration precedes IVD disease and has a similar etiopathogenesis. The term IVD herniation, which is defined as localized displacement of the intervertebral disc beyond the normal 3-dimensional anatomic limits of the disc, can be used to summarize the methods through which a degenerating disc can induce pain and neurologic impairments. Consequently, this can be roughly separated into 2 categories, each of which is connected to a different form of IVD degeneration: either the degenerating nucleus (type I) or the degenerating annulus (type II) protruding into the spinal canal (Jeffery NC, et al, 2013).

All dog breeds are susceptible to intervertebral disc herniation, but chondrodystrophic breeds including the Dachshund, Pekingese, French Bulldog, Basset Hound, Welsh Corgi etc. are those susceptible to be affected by it (Chai O et al, 2018).

Due to the distribution of locomotor forces provided from the pelvis to the vertebral column and the prevalence of congenital abnormalities in this intervertebral area, the lumbosacral region may be extremely vulnerable to IVD herniation (Jeffery NC, et al, 2013).

Similar to other studies, in the present paper chondrodystrophic breeds had higher prevalence for IVD herniation and subsequently pronounced muscle contracture. Obesity, genetic predisposition, and spinal kyphosis, in case of French bulldogs, should be taken into consideration as risk factors for development of clinically relevant thoracolumbar IVD herniation (Chai O et al, 2018; Inglez de Souza et al, 2018).

CONCLUSIONS

Muscles around V1 (Vertebra cranial to the compression site):

For M1R and M1L, the mean lengths in dogs with spinal cord compression are generally lower than those in the healthy dog.

For M2R, the mean length is significantly higher in dogs with spinal cord compression.

Muscles around V2 (Vertebra at the compression site):

For M1R, M1L, and M2R, the mean lengths are generally lower in dogs with spinal cord compression compared to the healthy dog.

Muscles around V3 (Vertebra caudal to the compression site):

For M1L, the mean length is lower in dogs with spinal cord compression.

For M2R, the mean length is higher in dogs with spinal cord compression.

Implications

Lower muscle lengths in dogs with spinal cord compression around V1 and V2 might indicate muscle atrophy or other structural changes due to compression (contraction).

Higher muscle lengths, particularly for M2R, might suggest compensatory hypertrophy or other adaptive responses.

All the patients with IVD herniation from this study presented muscular contracture in the compression area.

The limitation of this study was the relatively small number of patients, for further studies a bigger number of patients from a single breed is recommended in order to establish the normal and abnormal values.

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GONIOMETRIC MEASUREMENTS OF THE FEMORAL JOINT IN DOGS WITH HIP DYSPLASIA

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Abstract

Canine hip dysplasia is one of the most common orthopedic dieseases presents in most breeds of dogs but more prevalent in large breeds. Clinical examination through tests such as Ortolani, Barlow, and Bardens, alongside with radiological, tomographic, magnetic resonance, and ultrasonographic examinations, are the only methods for screening and diagnosing canine hip dysplasia. Canine hip dysplasia can lead to other musckuloskeletal diseases, with the most common ones being cruciate ligaments tear and spinal conditions such as IVDD or degenerative myelopathy. To assess hip changes, it is recommended that the patient to be heavily sedated, and the standard exposure will be with the hips in forced extension. Among knee conditions related to hip dysplasia, the most commonly reported are patellar luxation, partial cranial cruciate ligament rupture, and osteoarthritic changes. A study conducted on 65 dogs found concurrent hip dysplasia and patellar luxation in 28% of cases. Clinical goniometry is an objective and non-invasive method of measuring joint angles, allowing for the assessment of the normal range of joint movements. This method can indicate the severity of joint pathology. Currently, goniometry is often used in the field of imaging and it can be an important element in assessing the musculoskeletal system. The aim of this paper is to evaluate the effectiveness of goniometric measurements at the knee and hip joint levels in dogs with hip dysplasia and if there is a correlation. A total of 10 dogs from varying breeds of dogs aging from 1 to 3 years old, were deeply sedated in order to measure perform the x-rays in ventro-dorsal hip extended view for measuring the Norberg angle (Na), anatomic Latero-Proximal Femoral Angle (aLPFA), anatomic Latero-Distal Femoral Angle (aLDFA), mechanical Latero-Distal Femoral Angle (mLDFA), mechanical Latero-Proximal Femoral Angle (mLPFA). All patients were part of the clinical cases present in our department and the written consent of the owners was obtained for each dog. Norberg and femoral angles were measured within the local DICOM viewer and the statistical analysis was performed with IBM SPSS Statistics. All images were reviewed by an ECVDI resident, a radiology professor and a radiology intern. All patients within our study had a Na under the reference value of 105º. All the femoral angles were within limits regarding their normal values. We conclude that there is not a signicative relation between the Na and the goniometric values obtained, most likely because of other factors that are influencing these measurements (ex. breed variations, femoral slightly rotation, muscle contracture due to poor anesthesia management, other musculoskeletal genetic disorders etc.). Further research on a more relevant statistical sample is recommended, in order to determine the normal goniometric values and the changes that appear in case of hip dysplasia.

Keywords: hip dysplasia, Norberg angle, musculoskeletal diseases, goniometric measurements

Introduction

Canine hip dysplasia is a complex, polygenic condition found in most dog breeds but more prevalent in large breeds. It occurs in young dogs and can lead to the development of osteoarthritis in advanced cases (Mikkola L, 2021). Since 1935, when it was first described by Schnelle, the entire veterinary community has been and continues to be engaged in the battle to control and reduce the occurrence of canine hip dysplasia because it has a significant impact on the well-being of the patient and indirectly on the owner (King M, 2019; Mikkola L, 2020).

Clinical examination conducted through tests such as Ortolani, Barlow, and Bardens, along with radiological, tomographic, magnetic resonance, and ultrasonographic examinations, remains, for now, the only methods for screening and diagnosing canine hip dysplasia, especially in young patients (Butler and Gambino, 2017).

Canine hip dysplasia can also lead to comorbidities, with the most common ones being ruptured cruciate ligaments and spinal conditions such as IVDD or degenerative myelopathy (DeCamp et al., 2016).

To assess hip changes, it is recommended that the patient be sedated, and the standard exposure will be with the hips in forced extension. It is known that in this exposure, the femoral head is practically "forced" by joint tension elements to be positioned as deeply as possible in the acetabular cavity, a phenomenon known as 'screw-home.' This phenomenon can lead to false negative scores if exposure is performed on young patients and osteoarthritic phenomena are not visible, so the operator relies only on signs of subluxation as indicators of the severity of the condition (Soo and Worth, 2015).

Among knee conditions related to hip dysplasia, the most commonly reported are patellar luxation, partial cranial cruciate ligament rupture, and osteoarthritic changes. A study conducted on 65 dogs found concurrent hip dysplasia and patellar luxation in 28% of cases (Kalff, 2014).

Clinical goniometry is an objective and non-invasive method of measuring joint angles, allowing for the assessment of the normal range of joint movements (Petazzoni, Jaeger, 2008). This method is among the standard orthopedic examination techniques that help indicate the severity of joint pathology (Petazzoni, Jaeger, 2008). Currently, goniometry is often used in the field of imaging and is a fundamental element in assessing the musculoskeletal system.

The aim of this work is to evaluate the effectiveness of goniometric measurements at the knee and hip joint levels in the exposure used in the radiological diagnosis of hip dysplasia.

Materials and methods

The biological material consisted of 41 medium and large-sized dogs of different ages and sexes who presented themselves to the Department of Medical Imaging within USAMV Clu-Napoca with the purpose of being radiologically evaluated at the hip joint level. Among these dogs, some showed symptoms representative of hip dysplasia, while others were asymptomatic. Out of a total of 41 cases, 10 cases considered the most relevant to the topic were selected and analyzed. All selected dogs were radiologically diagnosed with hip dysplasia. The individuals included in the study were aged between 7 months and 3 years, and weighed between 20 kg and 52 kg. Auxiliary materials used were: X-ray machine, plastic restraint support and radiological positioning bags, Horos software program

The work protocol for all canine patients included in the study was the same: performing a radiological exposure in a ventrodorsal position with the hind limbs extended caudally, and the knee joints rotated inward so that the patella is centrally positioned on the limb (Morgan, Wind, Davidson, 2000). Some of the patients were placed in the veterinary plastic support that facilitates the correct position for evaluating the hip joint. The images were made so that the knee joint could also be studied. Measurements made were:

- Norberg angle: to evaluate the degree of hip dysplasia
- Goniometric measurements made on the femur in the frontal plane, based on the anatomical and mechanical axis of the limb: aLPFA (lateral-proximal anatomical femoral angle), mLDFA (lateral-distal anatomical femoral angle), mLPFA (lateral-proximal mechanical femoral angle), and mLDFA (lateral-distal mechanical femoral angle) (fig. 1).

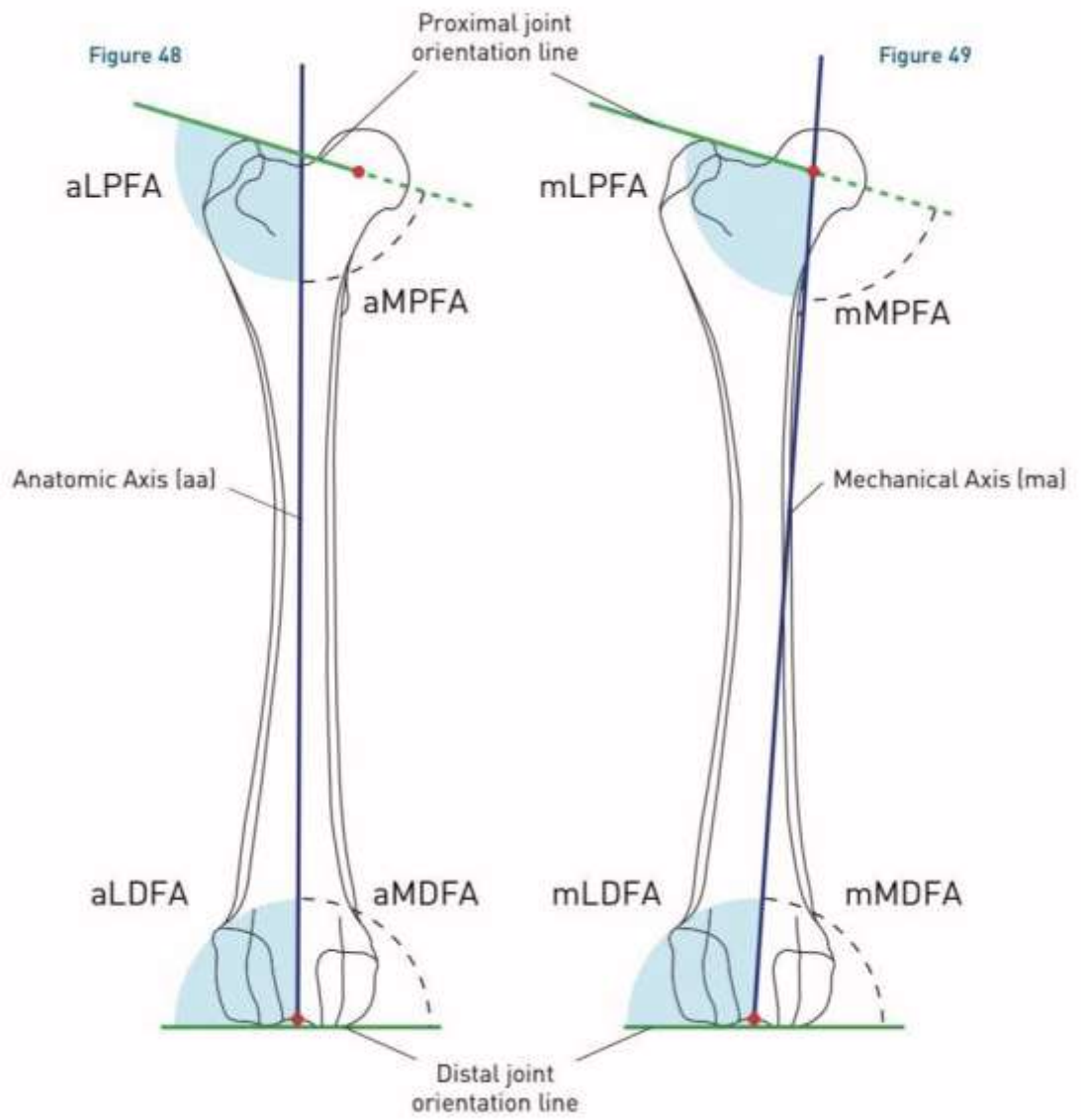


Fig. 1 Measuring methods for aLPFA, aLDFA, mLPFA, mLDFA

Petazzoni, Jaeger, Atlas of Clinical Goniometry and Radiographic Measurements of the Canine Pelvic Limb, 2008

Discussion

All determinations were made within the Horos program, a digital imaging program that allows the study of radiographs and the making of exact measurements. Auxiliary data about patients were obtained from the owners. All patients were part of the clinical cases present in our department (fig.2, fig. 3, fig. 4) and the written consent of the owners was obtained for each dog. Norberg and femoral angles were measured within the local DICOM viewer, and the statistical analysis was performed with IBM SPSS Statistics.

The statistical analysis follows the descriptive statistics of the obtained datas and the correlation between the Norberg angle and the values obtained for aLPFA/LDFA and mLPFA/LDFA for the examined patients.

The heatmap provides a visual representation of the strength and direction of the correlations. Positive values indicate a positive correlation, while negative values indicate a negative correlation.

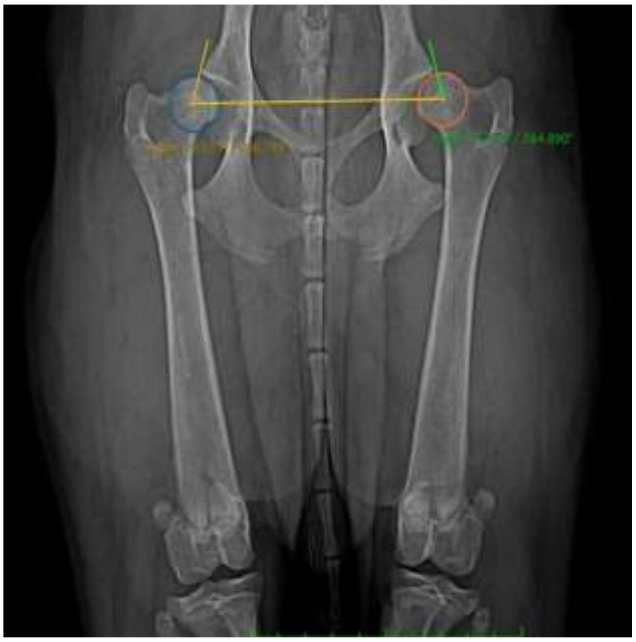


Fig. 2 Ventrodorsally exposure and Norberg angle measurements

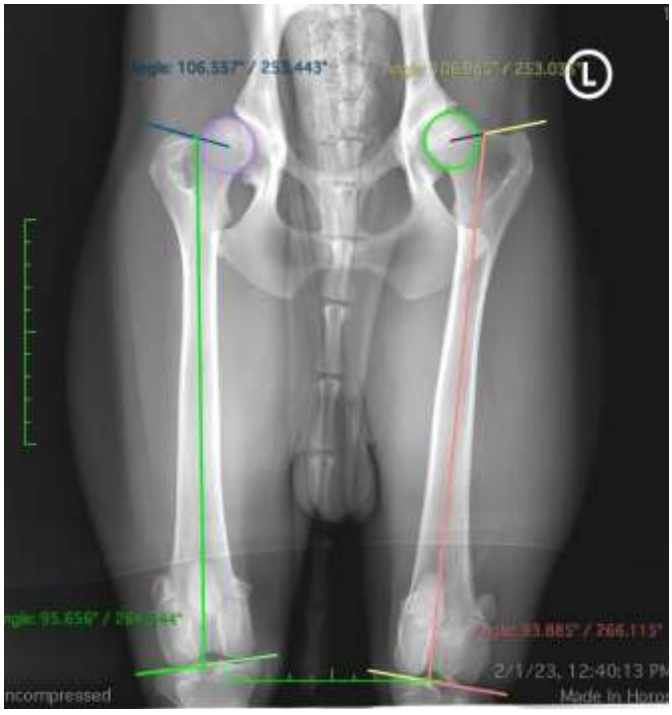


Fig. 3 Ventrodorsally exposure for aLPFA and aLDFA measurements

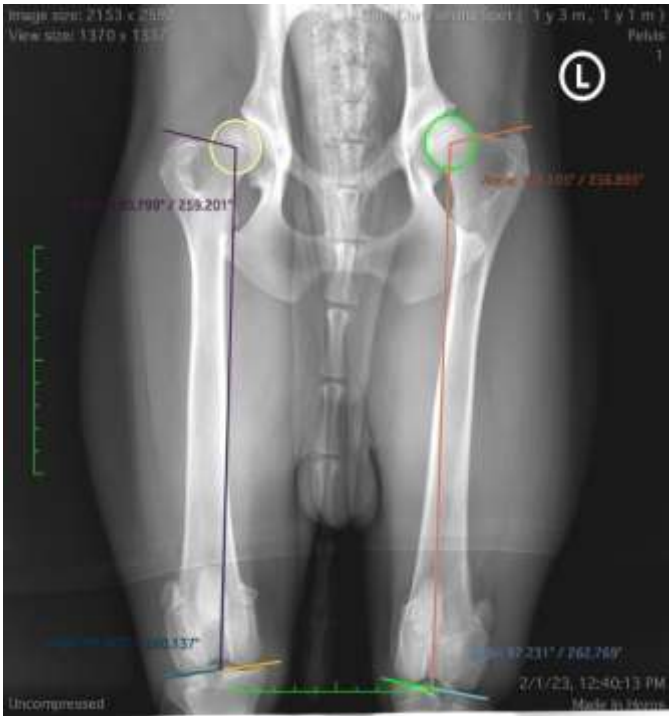
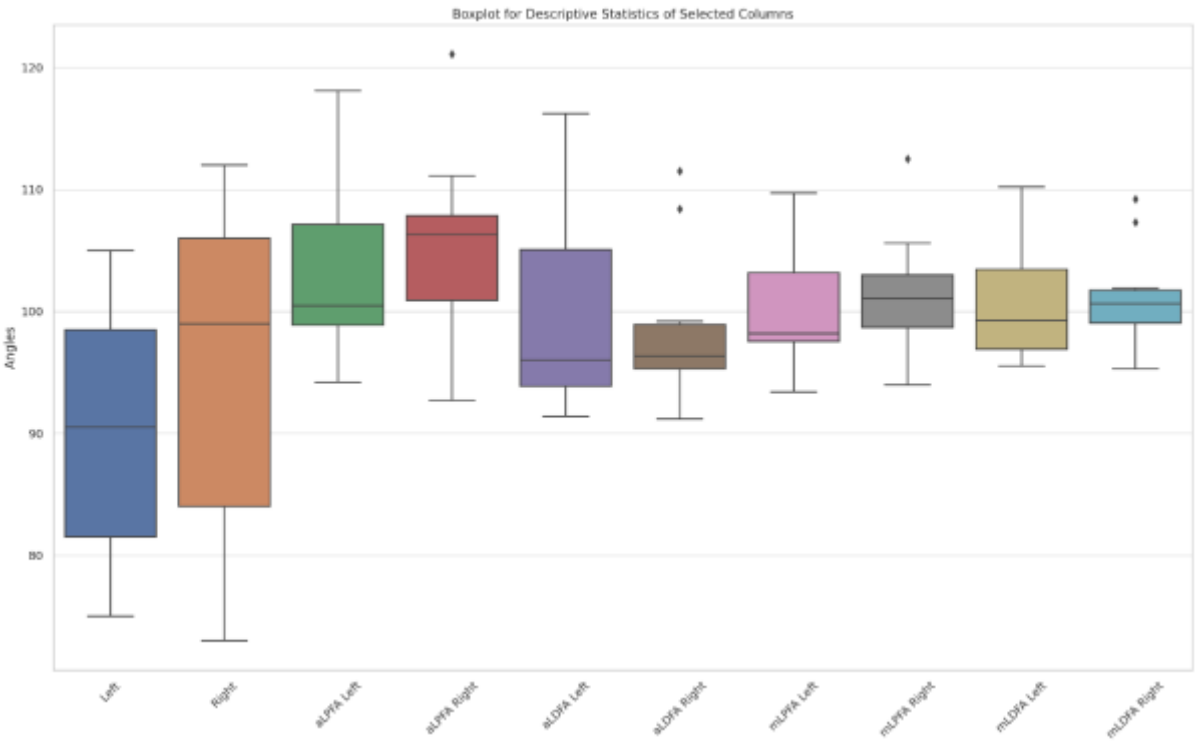


Fig. 4 Ventrodorsally exposure for mLPFA and mLDFA measurements



The central line in each box indicates the median.
The edges of the box indicate the interquartile range (IQR).
The whiskers extend to 1.5 * IQR.

Fig. 5 Boxplots for the Norberg angle and obtained measurements.

Table 1.

Descriptive statistics for the data's

Statistic	*Norberg Angle Left*	*Norberg Angle Right*	*aLPFA Left*	*aLPFA Right*	*aLDFA Left*	*aLDFA Right*	*mLPFA Left*	*mLPFA Right*	*mLDFA Left*	*mLDFA Right*
Mean	90.0	95.6	103.05	105.1	99.49	98.62	100.55	101.12	100.43	101.24
Std Dev	11.06	13.81	6.93	8.01	8.11	6.39	5.39	5.41	4.67	4.2
Min	75.0	73.0	94.2	92.7	91.4	91.2	93.4	94.0	95.5	95.3
25th Percentile	81.5	84.0	98.88	100.9	93.88	95.3	97.53	98.7	96.9	99.05
50th Percentile	90.5	99.0	100.45	106.3	96.0	96.35	98.2	101.05	99.25	100.65
Max	105.0	112.0	118.1	121.1	116.2	111.5	109.7	112.5	110.2	109.2

The Norberg angle for the right leg shows moderate to strong negative correlations with aLPFA Right and mLPFA Right.

The Norberg angle for the left leg does not exhibit strong correlations with any of the other angles, which suggests it may not be a good predictor based on these variables.

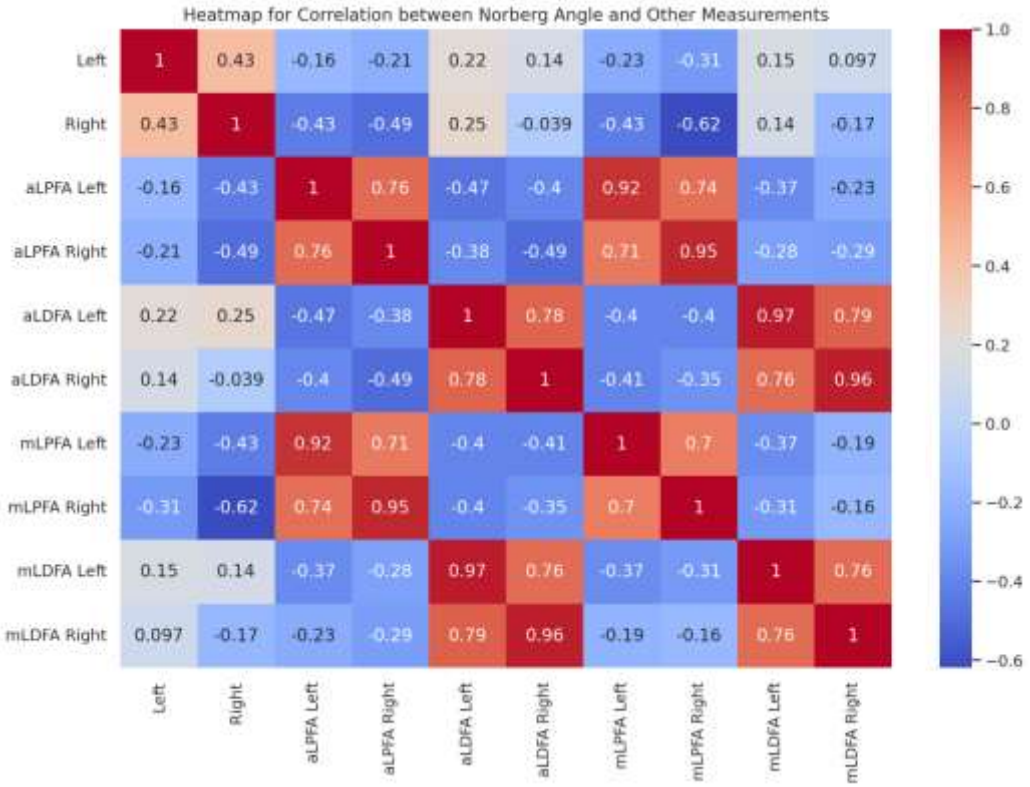


Fig. 6 Heatmap for correlation between Norberg angle and other measurements

Conclusions

In medical diagnostics, radiological examinations or other imaging techniques are essential for evaluating the normal appearance of hip and knee joints and for definitively diagnosing hip dysplasia. It is generally recommended that patients be sedated to ensure accurate positioning and yield the most relevant images. Goniometric measurements of aLPFA, aLDFA, mLPFA, and mLDFA obtained from conforming hip evaluations present a moderate level of relevance for assessing the normal alignment of the femur and adjacent joints. The study found no directly proportional relationship between the degree of hip dysplasia and the values of aLPFA, aLDFA, mLPFA, and mLDFA. It is also noted that dysplasia in one limb could potentially affect the alignment of the corresponding limb. Significant differences between the standard values proposed by different studies pose challenges in evaluating the normal frontal-plane alignment of the femur. Furthermore, breed-specific morphological and anatomical differences also influence the angles of aLPFA, aLDFA, mLPFA, and mLDFA. Other conditions that may or may not be correlated with coxofemoral dysplasia, such as patellar luxation, likewise affect these angle values. Finally, the study recommends further investigation of a statistically more representative sample concerning both the standard values of aLPFA, aLDFA, mLPFA, and mLDFA, as well as their variations in patients with coxofemoral dysplasia.

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ASSOCIATION BETWEEN FOOT SKIN TEMPERATURE (FST) AND LOCOMOTION SCORING (LS) IN DAIRY CATTLE

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Abstract

The health status of the hoof in dairy cattle is crucial for their overall well-being and productivity. Hoof diseases and lesions can lead to lameness, decreased milk production, and economic losses for dairy farmers. Traditional methods of assessing hoof health involve manual inspection and trimming, which can be time-consuming and subjective. This research article explores the potential use of a phone-connected infrared camera as a non-invasive and objective tool for assessing the health status of the hoof in dairy cattle, by investigating the association between foot skin temperature (FST) and locomotion scoring (LS) in a group of Romanian Black Spotted dairy cows. LS was carried out using the mobile app developed by the Wisconsin University (Locomotion Scorer). The thermograms were collected during afternoon milking and processed using the FLIR software. Overall, 73.9% of the cows were scored as non-lame, while 13.04% presented signs of foot lesions. The highest temperature observed in the interdigital area, in the lame group, by thermography, was T°=36.5°C. In conclusion, monitoring hoof health status in dairy cattle is essential for ensuring their well-being and productivity. The use of an infrared thermal camera for the assessment of foot surface temperature has shown promise as a noninvasive tool for evaluating hoof health.

Key words: infrared termography; lameness; locomotion score; dairy cattle

INTRODUCTION

Foot diseases, including foot rot and digital dermatitis, are prevalent in dairy cattle and can have a significant impact on animal welfare and productivity (Chapinal *et al*., 2013). Research efforts have focused on investigating the etiology, risk factors, diagnosis, and control of foot-related lameness in dairy cattle (Warema *et al*., 2021). It has been demonstrated that foot disorders in dairy cattle have a heritable component, indicating a genetic predisposition to these conditions Koenig et al, 2005; Oberbauer *et al*., 2013). Furthermore, modeling approaches have been used to assess the welfare impact of foot disorders in dairy cattle, highlighting the importance of pain intensity and clinical foot disorders in determining the welfare of the animals (Bruijnis *et al*., 2012).

Hoof health in dairy cattle is influenced by various factors, including nutrition and genetics. Langova *et al*., (2020) reviewed the impact of nutrients on hoof health in cattle and highlighted the role of minerals such as calcium, iron, copper, zinc, iodine, selenium, molybdenum, and chromium in hoof development and disease.

Genetic factors also play a significant role in hoof health. Genetic selection for hoof health traits can accelerate the rate of genetic gain in lameness in dairy cows. Ring *et al*., (2018) discussed the opportunities to enhance claw health through genetic selection and highlighted the importance of routine recording of claw health status for genetic evaluation. Furthermore, Solano *et al*., (2016) conducted a study to estimate the genetic parameters for hoof lesions and their relationship with feet and leg traits in Canadian Holstein cows. They found that there were significant genetic correlations between hoof lesions and feet and leg conformation traits, indicating that genetic selection for improved feet and leg conformation could lead to better hoof health

Monitoring hoof health status in dairy cattle is crucial for ensuring the well-being and productivity of the animals. Lameness, which is often caused by hoof disorders, is a complex condition that is challenging to detect and manage (Flowers and Weary, 2006). Traditional methods of assessing hoof health, such as locomotion scoring and visual inspection, have limitations in terms of accuracy and objectivity.

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One promising approach to assess hoof health is the use of infrared thermal cameras to measure foot surface temperature. This method allows for non-invasive and real-time monitoring of the temperature distribution on the hooves, which can provide valuable insights into the presence of hoof pathologies and potential lameness issues (Stokes *et al*, 2012; Warema *et al*., 2021).

This research article explores the potential use of a phone-connected infrared camera as a non-invasive and objective tool for assessing the health status of the hoof in dairy cattle, by investigating the association between foot skin temperature (FST) and locomotion scoring (LS) in dairy cattle.

MATERIAL AND METHOD

Data Collection

This study was carried out in March–April 2023 and data were collected from a number of 23 dairy Romanian Black Spotted cows reared in a semi-intensive system and milked twice per day, in a dairy farm located in the northeast of Romania. Prior to capturing the digital images, the hooves of the cattle were not subjected to any washing or cleaning procedures. This deliberate omission was made to ensure that the infrared thermography (IRT) devices used for lameness detection would not be compromised in their usefulness. A cut-off temperature value of 27 °C was used to define foot lesions of hind feet, as described previously by Stokes *et al*., 2012.

Clinical assessment of the locomotion score

Assessment of locomotion score required the observation of well-described gait and postural features, while cows were moving on a level surface. Visual observation of the cows was carried out after milking, because cows with claw horn lesions, such as sole ulcers, exhibit the most noticeable locomotor irregularities after milking. Scoring was carried out using the mobile app developed by the Wisconsin University (Locomotion Scorer). The software allows the locomotion scoring using a 4-category scale and provides images, video, and vocal explanations for evaluation. A graphical and numerical overview of the cows' scores was provided once the evaluation was completed (https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/locomotion-scorer/).

Foot skin temperature

The thermograms were generated using an infrared thermo-graphic phone-connected camera (Flir One Pro, Flir Systems). Thermograms of each cow's target region were acquired from 0.5 m distance during afternoon milking. The designated area was the heel bulbs and below in the hindfoot. The temperature reflected by the environment was 20 °C. The images were processed using the ThermaCAM software (FLIR Systems, USA).

Data processing

A total of 97 hind foot thermograms were acquired throughout the four days of data collection. However, upon analysis, it was determined that 7 of them did not meet the required quality standards for inclusion in this research. The exclusion was mostly attributed to the designated region not being completely visible or being blurry within the thermograms.

Statistical Analysis

The highest recorded temperature from each thermogram was exported to Microsoft Excel (Microsoft, USA) and associated with the locomotion score (LS) of the corresponding cow. Cows with LS values of 2 and 3 were categorized as lame. The statistical analysis was conducted using GraphPad Prism 9 software, developed by GraphPad Software in San Diego, CA, USA.

RESULTS AND DISCUSSIONS

In recent years, there has been a growing interest in the use of infrared thermography (IRT) as a non-invasive tool for assessing the health status of cattle, particularly hoof health. Several studies have demonstrated the potential of IRT in detecting various health conditions in cattle, such as udder health status, respiratory disease, lameness, and foot lesions. These cameras have been employed to study the relationship between an animal's physiology and surface temperature, as well as to analyze metabolic heat loss (Warema *et al*., 2021). By capturing thermal images of the hooves, it is possible to detect abnormalities in temperature distribution that may indicate the presence of foot diseases or injuries (Alsaaod and Büscher, 2012).

For instance, studies by Zaninelli *et al*., (2018), Byrne *et al*., (2018), and Alsaaod *et al*. (2015) have investigated the application of IRT in evaluating the health status of dairy cows, sheep, and cattle, respectively. These studies have shown promising results in using IRT for the early detection of health issues, including hoof lesions, lameness, and respiratory diseases.

Lameness in dairy cattle is a common health problem that affects animal welfare, milk production, and longevity. One of the traditional methods of assessing lameness is visual observation grading, which uses a locomotion score to compare healthy and unhealthy cows on a scale of 0 to 4, with 0 being a healthy cow and 5 being a severely sick cow.

In our study, 73.91% of the analyzed cows did not exhibit any signs of foot injuries and were

graded as normal, with score 0, white 13.04 of the cows received 2 score, indicating the debut of hoof lessions (Table 1).

Although the assessment of locomotion score is a method that has been routinely used in dairy farms globally, it relies on subjective gait scoring systems, which can be time-consuming and prone to human error.

Table 1.

Distribution of locomotion score and their percentage in the analyzed group

	No. of scored cows	***%***
1- Walks with even weight bearing and rhythm on all four feet, with a flat back. Long fluid strides possible	17	
2 - Steps uneven or strides shortened, but affected limb or limbs not immediately identifiable.	9	
3 - Uneven weight bearing on a limb is immediately identifiable and/or obviously shortened strides (usually with an arch to the center of the back).	6	
4 - Unable to walk as fast as a brisk human pace coupled with uneven weight bearing and shortened stride, with a back arch.	3	
Total	***45***	

Table 2.

Descriptive statistics of the results obtained from hoof assessment using thermography

Category	***Mean***	***Standard Deviation***	***Min value***	***Max value***
Healthy cows group	30.8 °C	2.7 °C	26.7 °C	33.5 °C
Lame cows group	35.3 °C	3.6 °C	34.1 °C	36.1 °C

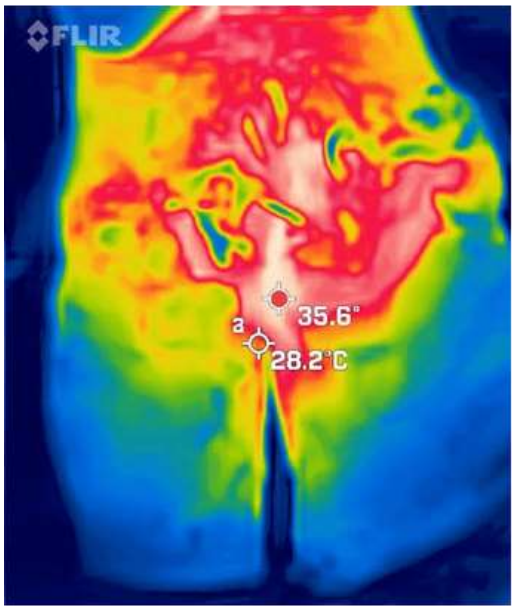

Figure 1 Infrared Thermal image of foot of the dairy cow exhibiting increased temperature in the interdigital area (T°-36.5°C) in a cows with score 3 (locomotion score). The red and blue hues of the scale correspond to the highest and lowest temperatures, respectivel

Our preliminary study showed a linear association between individual cow locomotion score and foot skin temperature. Cows with locomotion score above 2 and 3 exhibited higher skin surface temperature as compared to cows with score 1.

In recent years, there has been a growing interest in using objective measures, such as infrared thermography, to enhance lameness detection. Studies have shown that handheld infrared thermometers can be used to optimize lameness detection by analyzing foot-surface

temperatures and temperature differences between the hind feet of individual cows (Gelasakis *et al.*, 2021).

Our findings suggest that even a low-cost thermal imaging device, such as the one used in the study has the potential to serve as a tool for detecting lameness. If practitioners and vets were to use these devices more often, it might lead to higher rates of lameness identification and ultimately improve animal welfare.

CONCLUSIONS

In conclusion, monitoring hoof health status in dairy cattle is essential for ensuring their well-being and productivity. The use of an infrared thermal camera for the assessment of foot surface temperature has shown promise as a noninvasive tool for evaluating hoof health. Infrared thermography (IRT) can detect surface heat emitted as infrared radiation and generate pictorial images without causing radiation exposure. Several studies have demonstrated the potential of IRT for the detection of lameness and other hoof lesions in dairy cattle. Hoof health is influenced by various factors, including nutrition and genetics.

Minerals and genetic selection for improved feet and leg conformation have been identified as important factors in hoof health. Other diagnostic methods, such as computerized claw trimming database programs, have also been used for monitoring hoof health in dairy herds. Overall, the combination of IRT and other diagnostic methods can provide valuable insights into the hoof health status of dairy cattle.

Further research is needed to validate the effectiveness and reliability of this approach in practical settings.

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IMPACT OF A2 MILK ON HUMAN HEALTH AND THE DAIRY INDUSTRY - A REVIEW

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Abstract: In recent years, a new type of cow's milk, called "A2 milk," has been introduced to the market. This type of milk was first marketed in New Zealand and has since gained a presence in the markets of several countries. It mainly contains two types of β-casein: the A1 and A2 variants. In recent years, researchers have studied the possible implications of the composition of the β-casein protein fraction for the manifestation of a new intolerance: milk protein intolerance. Casein is the main component of milk proteins, of which approximately 30-35% is beta-casein. A2 beta-casein has proline at position 67 of the protein amino acid chain, while A1 beta-casein has histidine at that position; this is associated with a possibility of gastrointestinal discomfort due to β-casomorphin-7 (BCM-7) released during gastrointestinal digestion. The purpose of this review is to provide an update on the impact of A2 milk on human health as well as on its many technological qualities for the production of dairy products with improved health benefits for consumers.

Key words: bovine, A1 and A2 milk, human health, dairy products.

INTRODUCTION

Milk is an essential nutritional food product for a significant number of people. It is obtained from the secretion of mammary gland of mammals and is mostly made up of water (about 87%), but it also contains lactose, triglycerides, high-quality proteins, minerals (calcium, magnesium, selenium), and vitamins (riboflavin, vitamin B12, pantothenic acid). Milk proteins are classified into three types based on their solubility potential: caseins (approximately 80%), whey proteins, and fat globule membrane proteins (Jiménez-Montenegro *et al*, 2022; Priyadarshini *et a*l, 2018).

The most prevalent protein is beta-casein, which has a good content of amino acids. The bovine beta-casein gene can undergo twelve different mutations; A1 and A2 are the most common genetic alterations. A single nucleotide mutation causes the A1 and A2 forms of beta-casein to change at amino acid position 67, where A2 milk contains proline and A1 milk contains histidine (Figure 1).

This polymorphism results in a significant conformational alteration in the secondary structure of the expressed casein protein. The bioactive peptide beta casomorphin 7 (BCM7) is produced when the A1 version of β-casein (raw or processed milk) is digested by the digestive tract using proteolytic enzymes (Sodhi *et al*, 2012; Petrat-Melin *et al*, 2015). Due to increased price volatility, rising production costs, and a recent deregulation process brought about by the elimination of milk quotas, the dairy industry in Europe has been engulfed in a serious crisis in recent years (Beldycka-Bórawska *et al*, 2021; Pouch *et al*, 2018). Furthermore, plant-based milk alternatives, which are frequently promoted as a healthier, more environmentally friendly, and animal-friendly option for bovine milk, are replacing their consumption in various consumer groups. The dairy industry must figure out how to become more profitable in this situation (Beldycka-Bórawska *et al*, 2021). One option is to include milk in the diets of consumers who do not currently eat it. In this instance, those who have unfavorable responses after consuming milk and dairy products are one of the key segments of the population that could increase their consumption of milk and dairy products. Lactose malabsorption, which affects roughly 65% of the adult population worldwide (Semwal *et al*, 2022), is the primary cause of digestive problems. Lactose-intolerant people have a variety of digestive symptoms after consuming milk, including abdominal pain, bloating, changes in stool frequency, and changes in stool consistency. Another strategy for dairy profitability is to look for new products with higher added value, such as milk or dairy products with health advantages (Fernández-Rico *et al*, 2022).

The purpose of this review is to provide an update on the impact of A2 milk on human health as well

as on its technological qualities for obtaining dairy products.

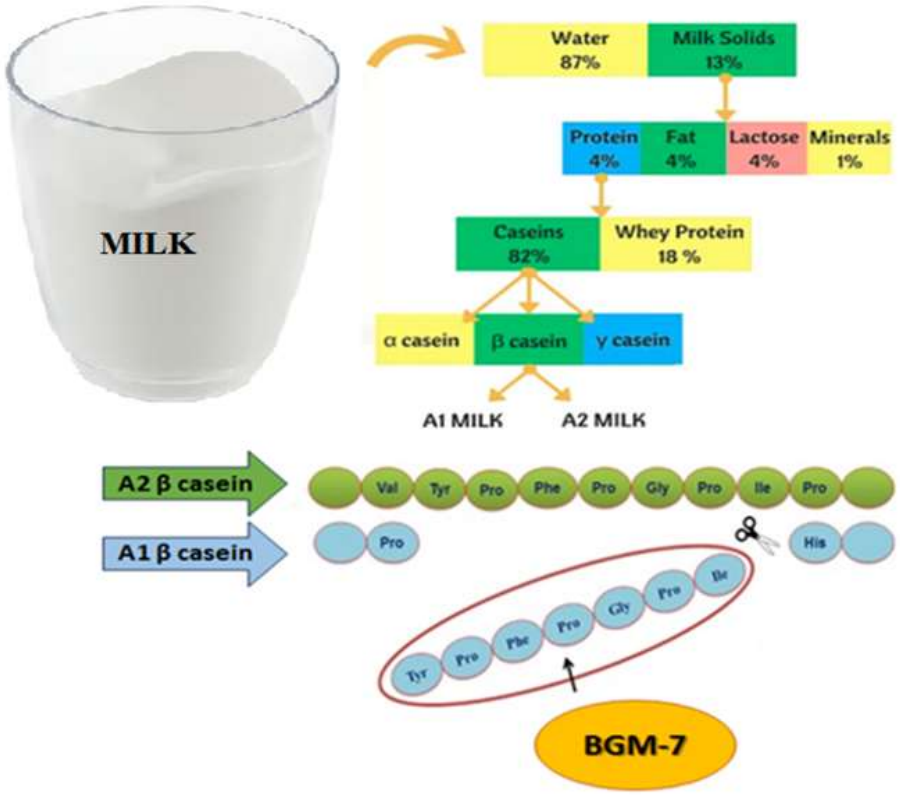

Figure 1 The composition of cow's milk and formation of β-casomorphin-7

The differences between A1 and A2 milk beta-casein

A2 milk is cow's milk that includes exclusively the A2 beta-casein protein type. According to the literature, cows generated only the A2 beta-casein protein and not the A1 beta-casein protein more than 10,000 years ago, before they were domesticated. A natural single-gene mutation occurred at Holsteins approximately 8,000 years ago, leading to the synthesis of the A1 beta-casein protein in this breed. Mutations in the beta-casein gene resulted in 12 genetic variations, the most prevalent of which are A1 and A2. Because Holsteins are used to increase the production of other breeds genetically, the mutation was passed on to too many other breeds (Sodhi *et al*, 2012).

The A1 beta-casein form gradually became dominant in milk. While dairy herds in much of Asia, Africa, and parts of Southern Europe still have a large proportion of A2 milk-producing cows, the A1 variant of the protein is more widespread in cattle in the Western world (Jiménez-Montenegro *et al*, 2022).

On chromosome 6 of the bovine genome is the beta-casein. The A1 allele of the gene is noted if β has the CCT codon that codes for the proline located at the 67th position in the casein chain and if the gene in this locus encodes histidine via the CAT code rather than the CCT code from the A2 allele. Because of this, bovines with the A1A1 genotype make A1 milk, or, to put it another way, histidine is found at position 67 of β casein.

Animals with A1A2 genotypes produce both A1 milk, or milk containing "histidine" and β casein, and A2 milk, or milk containing "proline" and β casein. A2 milk is the term used to describe the milk produced by animals carrying the A2A2 gene (Demirel and Çak, 2018).

The beta-casein gene, which is located on chromosome 6, regulates whether cows produce A1 or A2 milk. Cows have historically produced A2 milk, which is considered safe and healthy. The 67th amino acid in the beta-casein gene was altered from proline (A2 allele) to histidine (A1 allele). A cow only has two copies of the beta-casein gene. As a result, she could have an A2A2 homozygous genotype, an A1A2 heterozygous genotype, or an A1A1 homozygous genotype.

The alleles do not have a dominant-recessive connection, meaning they are co-dominant. As a result, an A1A2 cow will generate equal amounts of A1 and A2 beta casein alleles. A2A2 cows only generate A2 beta-casein, whereas A1A1 cows only produce A1 beta-casein. A2A2 cows pass on the A2 allele to their offspring, while A1A1 cows pass on the A1 allele, and A1A2 cows have an equal probability of passing on either genes. A2A2 cows can be obtained using sperm from bulls of the A2A2 genotype (Sridharan *et al*, 2020).

The Effects of beta casomorphin -7 (BCM7) and β-Caseins from milk on Human Health

Consumption of -casein A1 has been linked to the development of diabetes mellitus, according to a study conducted on non-obese diabetics. BCM-7 affects several opioids in adults. BCM-7 has been linked to immune system inhibition, increased risk of ***type 1 diabetes, arteriosclerosis, coronary heart disease, and sudden infant death syndrome***.

Numerous neurological conditions, including schizophrenia and autism, have also been connected to it. Lactose intolerance is usually blamed for stomach discomfort after consuming dairy receptors in the endocrine, neurological, and immune systems (Şahin *et al*, 2018). Infants are more likely to absorb BCM-7 because of their underdeveloped gastrointestinal tracts compared to lactose, which impacts digestion and creates symptoms similar to lactose intolerance in some people products, such as gas, bloating, and diarrhea (Miranda *et al*, 2015; Fernández-Rico *et al*, 2022).

It is also recognized to be an oxidant of low-density lipoproteins (LDL), and it is thought that LDL oxidation plays a significant role in the development of arterial plaque. To confirm the scope and kind of BCM7 interactions with the human gastrointestinal tract and entire organism, more investigation is required for animal testing and data collection on human patients experiencing issues linked to A1/A2 beta-casein milk consumption is needed for this (Fernández-Rico *et al*, 2022).

Technological characteristics of A2 milk and impact in dairy industry

Milk from cows has been consumed by humans for thousands of years, but as dairy production and consumption have increased recently, so too have health problems related to allergies and intolerances to the protein found in cow's milk (Parashar *et al*, 2015; Rangel *et al*, 2016).

Cow's milk protein allergy is an adverse immunological reaction when the body is exposed to dietary antigens in cow's milk (Jiménez-Montenegro *et al*, 2022).

Researchers have been examining the potential effects of the -casein protein fraction's composition on the emergence of a novel sensitivity known as milk protein intolerance in the last several years. Many people who think they are lactose intolerant may not truly be experiencing this issue (Rangel *et al*, 2016).

Although the worldwide consumption of milk and dairy products is constantly increasing and is expected to continue increasing over the next decade, milk consumption has decreased significantly since the 1970s in specific geographical areas, such the United States and the European Union. Thus, the dairy industry has tried to be creative and develop new products to increase consumption. In 2003, the A2 Milk Company Limited emerged in New Zealand, commercializing both milk and dairy products (cheeses, yogurts, or creams) free of the A1 variant of β-casein. A2 milk strongly entered the market in this country and covered almost 10% of the milk market in Australia. Given the possible benefits of A2 milk for human health, in addition to avoiding the negative effects of β-casein A1, many farmers around the world have switched to A2 milk production (Mayer *et al*, 2021). This successful market trend has spread to other geographic areas, such as North America, Europe, and China. Consequently, other companies dedicated to the commercialization of semen for dairy farms have introduced the A2/A2 genotype in their sire directories as a characteristic of interest and added value for their animals. According to researchers, A2 milk as smaller fat globule diameters and higher polyunsaturated fatty acid content than A1 milk (Perna *et al*, 2016).

Milk fatty acids and fat globule size have an impact on the physicochemical, nutritional, and sensory aspects of milk and milk products. Casein polymorphism has a profound influence on the morphometric properties and fatty acid composition of milk, as evidenced by the large differences discovered among milk casein haplotypes (Fernández-Rico *et al*, 2022; Albarella *et al*, 2020). These findings are intriguing since globule size differential affects renneting, cheese texture, color, flavor, and butter texture. Some authors discovered higher rates of fat in A2 animals than in A1 genotype cows generated by the same dairy cow breed and in the same environment. Other major dairy industry processes for which a different activity was recorded from A1 and A2 milk include emulsion and foaming capacities, albeit the results published are not enough (Nguyen *et al*, 2018;

Delgado Teixeira, 2021).

Darewicz and Dziuba studied the emulsion qualities of milk containing several types of β-casein (Darewicz and Dziuba, 2007). They came to the conclusion that while A2 milk formed emulsions more effectively than A1 milk, they were not as stable as those made with the A1 and B varieties. Differences in the emulsifying abilities of the A1 and B variations are also partly due to their better-ordered structures in the absorbed state than the A2 variant (Fernández-Rico *et al*, 2022).

CONCLUSIONS

Some research supports Beta-CasoMorphin-7 (BCM7) to be a risk factor for human health bas it can potentially affect numerous opioid receptors in the nervous, endocrine and immune system.

Cows' milk A2 differs from A1 milk in terms of technological qualities. Proline in A2 is a major impact on the hydrophobicity of the protein, leading to less ordered structures that impact the size of casein micelles, emulsifying and foaming qualities, and the production of curd and rennet.

Reviews were undertaken on a regular basis, and they found that more research is needed to determine whether A2-related things are preferable to A1. Despite an increase in publications from numerous universities and academic sectors around the world in recent years, human clinical trials remain scarce.

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WHEN GALLBLADDER OEDEMA COMES AS A CHRONIC DISEASE – DIAGNOSIS AND TREATMENT

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Abstract

Diagnosis of the gallbladder oedema is difficult during the clinical examination because the clinical signs are not specific for this disease. The causes of this pathology are multiple and can be divided into emergencies (such as anaphylactic shock, right-sided hearth failure, pericardial effusion) and chronic pathologies (such cholecystitis, pancreatitis or immune-mediated hemolytic anemia). Imagistics methods complete the information of the clinical exam and ultrasound is considered the golden standard for diagnosing this pathology. Normally, at the ultrasound exam, the gallbladder has a hyperechoic wall, with a diameter of 2-3 mm; while in case of gallbladder oedema, the wall is thick and has a triple stratification, with 2 hyperechoic rows, separated by a hypoechoic line. This pathognomonic description of the gallbladder oedema is also known as the 'Halo Sign' or 'Double Rim Effect'. Making a correct differential for the appearance of the sign is vital for the outcome of the case. The main focus is to exclude every emergency cause in order to start treating the chronic pathologies. This paper presents the pathologies leading to gallbladder oedema, met at the Radiology and Emergencies Departments from the Faculty of Veterinary Medicine Iasi during the period march 2020 – may 2022. The clinical case presented was diagnosed and treated at the Center of Endoscopy and Minimally Invasive Surgery Bucharest.

Key words: Gallbladder Oedema, Ultrasound, Surgical treatment

Gallbladder oedema clinical signs are not specific having a large range; they can include: vomiting, abdominal pain, diarrhea, effusion, anemia. The causes of this gallbladder wall lesions are multiple and can be divided into emergencies and non-emergencies as followed:

Table 1

Emergencies that can cause gallbladder oedema	Non-emergencies that can cause gallbladder oedema
Anaphylaxis	Cholecystitis
Right-sided hearth failure	Pancreatitis
Pericardial effusion	Hypoalbuminemia
	Immune-mediated hemolytic anemia

Ultrasound is more used in veterinary medicine and is considered the golden standard for diagnosing the gallbladder oedema. Normally, at the ultrasound exam, the gallbladder has a hyperechoic wall, with a diameter of 2-3 mm (figure 1); while in case of gallbladder oedema, the wall is thick and has a triple stratification, with 2 hyperechoic rows, separated by a hypoechoic line. This description of the gallbladder oedema is also known as the 'Halo Sign' or 'Double Rim Effect'(figure 2). Other imagistic exams that can help us view the oedema is represented by

computer tomography (Lisciandro G.R., Gambino J., Lisciandro S.C, 2021).

Gallbladder oedema is most seen in cases of anaphylaxis. It is also the organ considered to be the canine shock organ because of the high concentration of mast cells from the level of the liver and gastrointestinal tract. The cause of the oedema is the massive release of histamine in the portal circulation; this will result into hepatic venous constriction and congestion (Lisciandro G.R., 2021).

Other emergencies where we can find this sign are represented by right-hearth failure or pericardial effusion. In this cases, there is a mechanical obstruction of blood flow into the right atrium, that with lead towards a backflow at the level of the vena cava and distension at this level. This will finally result in hepatic venous congestion and wall oedema (Lisciandro G.R., 2021).

On the other hand, some of the most common non-emergencies that can cause this sign are represented by hypoalbuminemia, cholecystitis and pancreatitis. Hypoalbuminemia and its relationship to gallbladder wall oedema is still under study but recent papers show that the the level of albumins in plasma or serum is not associated with the gallbladder imaging, having a delay of 48 hours and sometimes other causes may be responsible for the imaging changes (Murakami M. et all, 2023). Colecystitis can sometimes, in chronic cases, give ultrasound changes and a small percentage have been seen with wall thickening or a fake aspect of oedema (Mitsui I., Ohtsuki S., Uchida K., 2021).

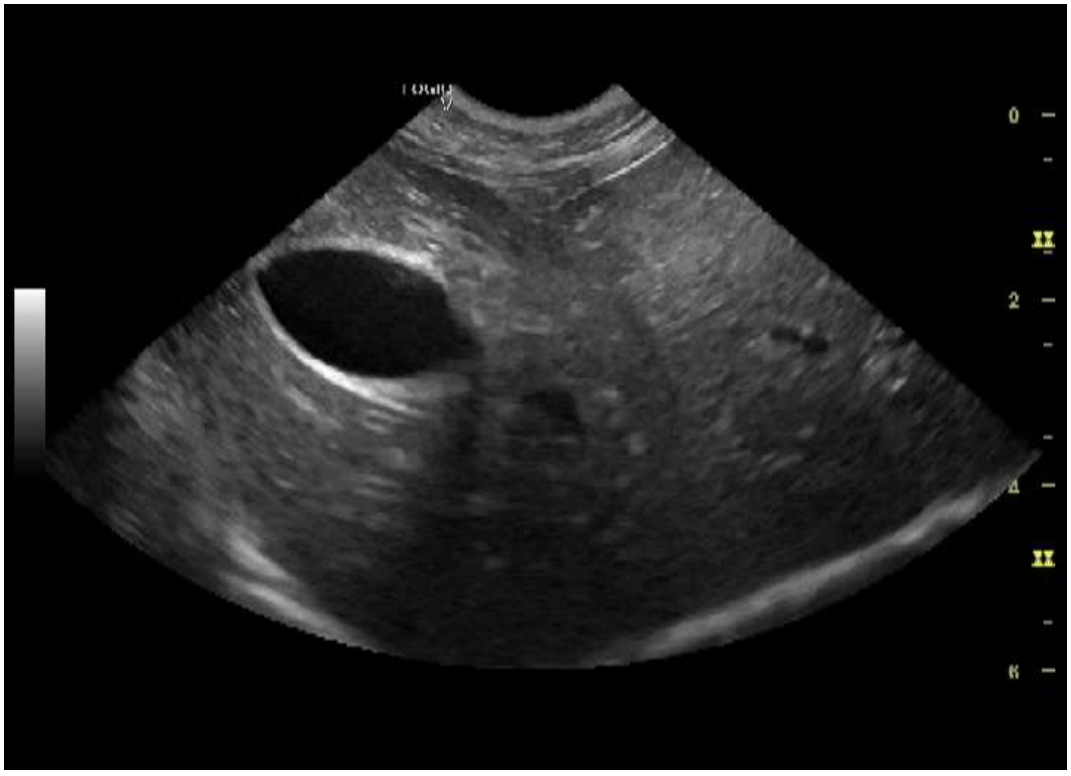

Figure 1. Normal gallbladder on ultrasound

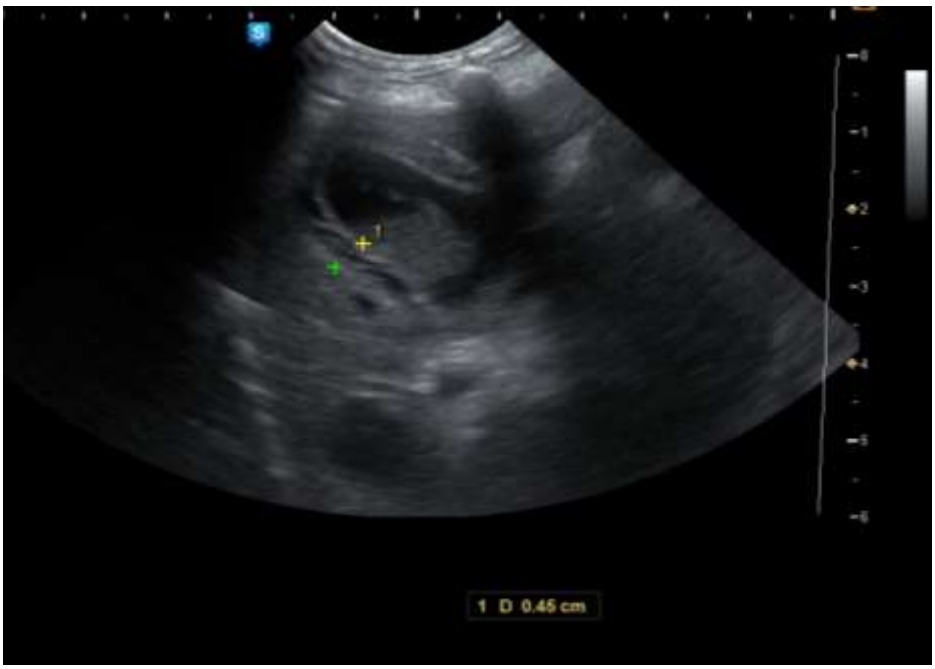

Figure 2. Gallbladder wall oedema, with the thickening of the wall (0.45 cm) and sludge

A treatment for this pathologies can be represented by cholecystectomy. Classic and laparoscopic cholecystectomy are both used in dogs but nowadays the laparoscopic method is being used more and more for its better outcome (Kanai H. et all, 2018).This surgeries can be seen also in human medicine in cases of acute cholecystitis or gallbladder stones; laparoscopic surgery has become a golden standard in human medicine (Acar T. et all, 2017; Van Breda Vriesman A. et all, 2007).

CASE SUMARY

A 3 year old F, Yorkshire Terrier presented to the clinic with vomiting, abdominal pain and apathy. There were no other findings at the clinical examination so an abdominal ultrasound and blood analyses were performed. The abdominal ultrasound showed an inflammation at the level of the pancreas, some biliary sludge and a thickened gallbladder wall with a suspicion of oedema (figure 3). The blood analyses showed a normal complete blood count, hypoalbuminemia (2.1 g/dL, normal range from 3.2 to 4.1 g/dL), increased ALP (253 U/dL, normal range from 7 to 115 U/dL), increased CRP (107 mg/L, normal range from 0 to 20 mg/L) and increased CPL (over 1000 µg/L, where over 600 µg/L is compatible with pancreatitis).

The dog was admitted for fluid therapy and supportive care.

The medication treatment with fluid therapy was given for 3 days without any improvement signs. The ultrasound was repeated the 2nd day without any major changes and the 3rd day again when the gallbladder oedema was more pronounced (figure 4).

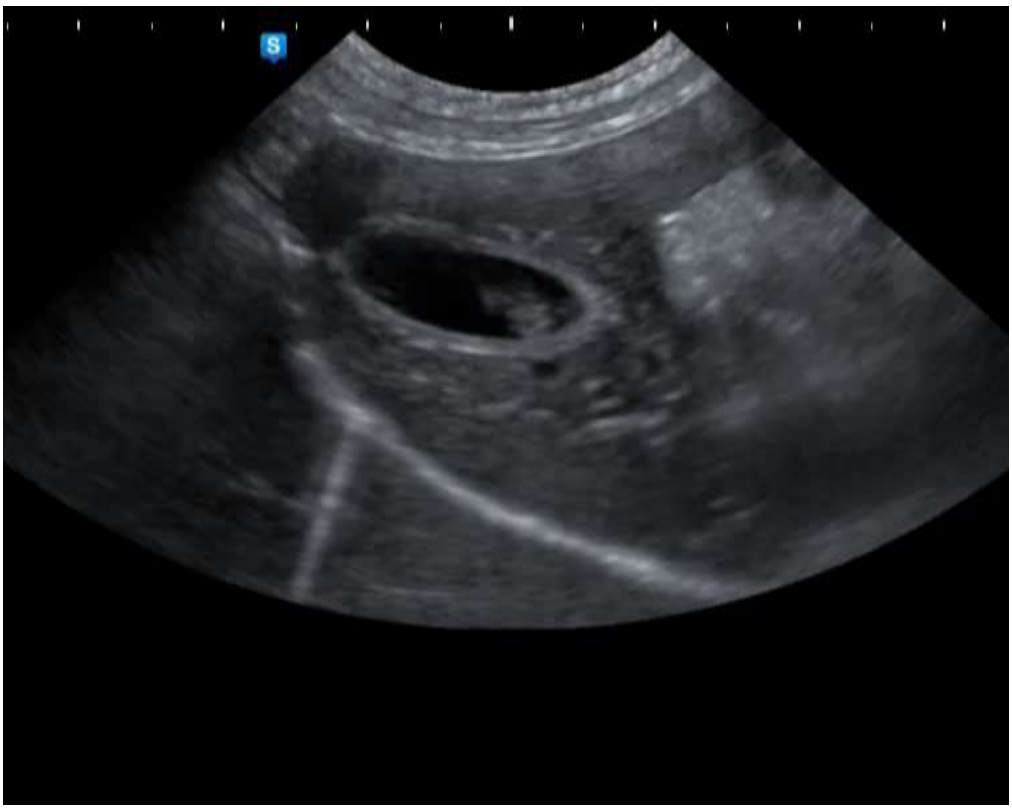

Figure 3. Ultrasound aspect of gallbladder sludge with thickened wall and a suspicion of oedema

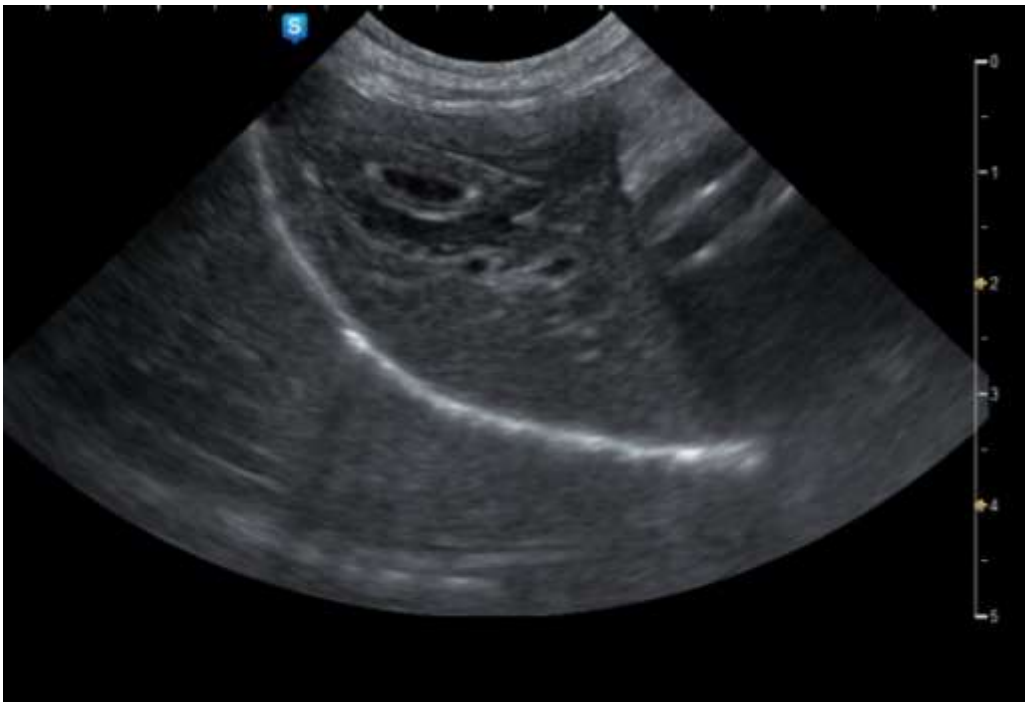

Figure 4. Ultrasound aspect of wall oedema

In the 3rd day the analysis were repeated showing changes on the CBC: WBC increased to 23.4 10^9/l (ranging from 6 to 17 10^9 /I) and RBC decreased to 3.7 10^{12}/l (ranging from 5.5 to 8.5 10^{12}/l). The biochemistry showed a lower albumin (1.7 g/dL) and no other significant changes from the 1st day.

In this case the decision was of advanced imagistic exams (Computer tomography) and laparoscopic cholecystectomy. Computer tomography showed the gallbladder wall changes

(figure 5 and 6) without any other significant findings.

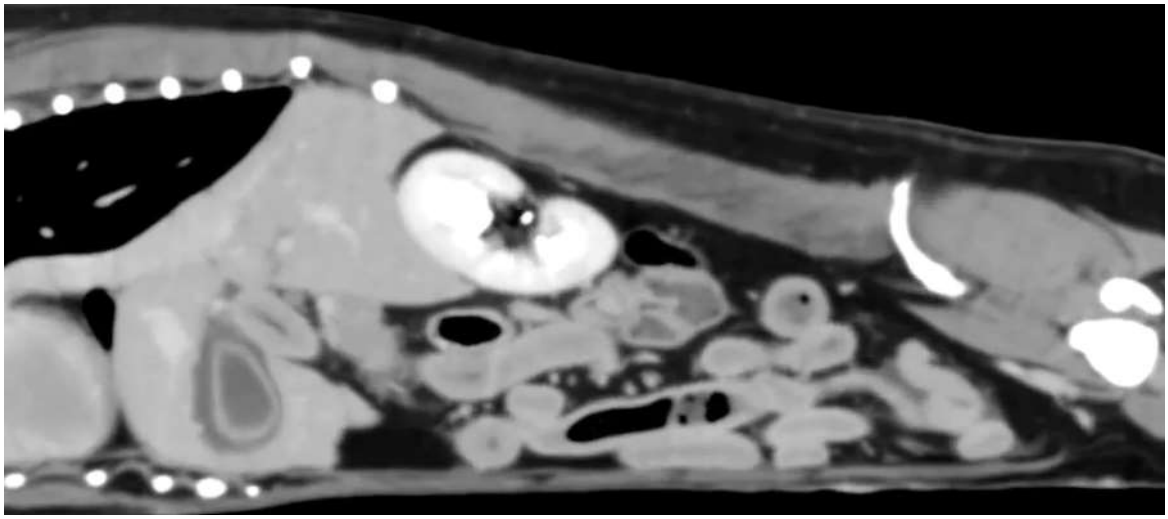

Figure 5. CT thickening and oedema of the gallbladder wall (Sagital)

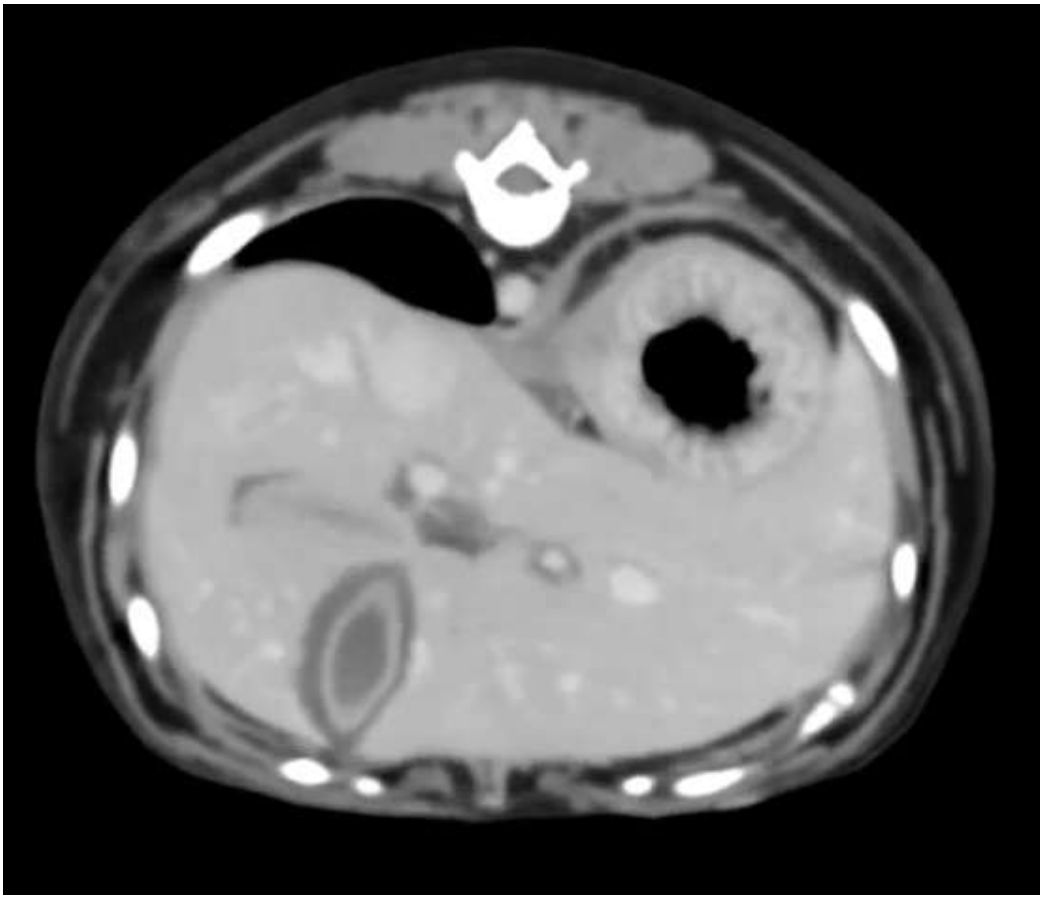

Figure 6. CT thickening and oedema of the gallbladder wall (Axial)

Laparoscopic cholecystectomy was started but because of intra-operatory complications, it was decided to convert to classical surgery (figure 7). The gallbladder (figure 8) was sent to the laboratory for histopathology where the result was chronic limfoplasmocitar cholecystitis and to bacteriology where the result was negativ.

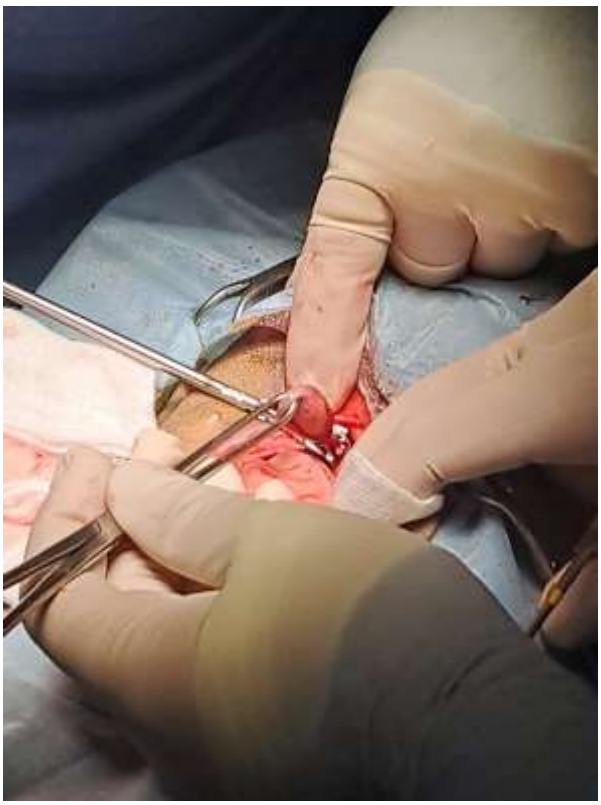

Figure 7. Surgical aproach of the gallbladder

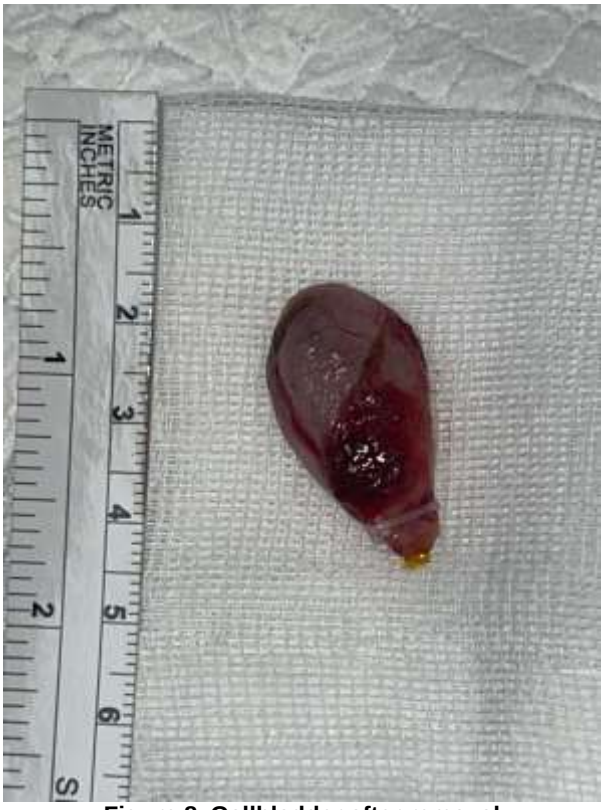

Figure 8. Gallbladder after removal

The next day after the surgery, the clinical signs started to regres and the pacient was fed with a syringe. The 2nd day after the surgery the pacient started eating by himself and on the 4th day his clinical state came back to normal, without any more vomiting, apathia or abdominal pain.

The ultrasound exams that followed showed only small signs of peritoneal inflamation

and a small quantity of free liquid. The blood analysis showed values getting slighly better such as CPL being 600 µg/L, CRP 70 mg/L or albumin 2.7 g/dL.

The 5th day after surgery the dog was discharged and at the 2 months re-check he was in normal clinical state and all the blood values came back to normal.

DISCUSSIONS

Ultrasound is an exam that can bring us many crucial information of the changes the patient suffers. It's advantages are the cost effectivness, the reduced time of a full abdominal exam or A FAST depending on the patients status and the low number of resources needed in realizing it. It is always indicated in such cases to have a serial ultrasound (every 4 or 8 hours when posible) to make the difference between an emergency and a non-emergency pathology.

From the sumary above, we can see that gallbladder oedema is a non specific sign without history of the patient and serial abdominal ultrasound. Cases with this kind of clinical signs need constant monitoring and extra laboratory or imaging exams for a better aproach. Only after 48 or 72 hours we can decide what are the best steps and what is the best treatement for the patient.

The patient presented the gallbladder oedema sign at 48 hours from admition. This showes us that altough the clinical signs and blood analyses are modified, the ultrasound changes are not always present and need time and multiple checks for them to apear. Cases like this can be missed if the ultrasound serial exam ins't taken into account.

Aproaching such cases with a good medical protocol is key in treating the patient. We need to have succesive steps in diagnosis and treatement. For this particular case gathering the information and starting a symptomatic treatement for stabilizing the patient had a major importance. Without these 1st steps, we couldn't reach the advance imagistic exams, surgery and having a final dignosis and also a treatment of the disease.

Clinical signs, ultrasound or blood analyses can only help us towards a presumtive diagnosis; the final diagnosis can only be made by laboratory exams suchs as histopatology from the walls of the gallbladder or in some cases bacteriological exams from the content. In this case we can see that the reasons of all this clinical signs was only known for sure after the histopathological dignosis of chronic limfoplasmocitar cholecystitis.

CONCLUSIONS

Gallbladder oedema can be caused by a large number of pathologies; an important aspect is to make the difference between emergency and non-emergency. The large range of clinical signs that can appear in this cases need a good protocol for the medic to eliminate every factor from the presumptive diagnosis. Most of the time when we encounter this sign or clinical signs associated to it, serial ultrasound examination is the key of evaluating the dynamic of the pathology.

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THE IMPORTANCE OF CANINE PANCREATIC LIPASE IN RELATION TO ULTRASOUND EXAMINATION

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Abstract

Pancreatitis represents the most often met pathology of the exocrine pancreas. It can be acute or chronic depending on the percentage of lesions to the parenchyma. The causes of the pathology aren't fully understood yet but it has been observed that inadequate nutrition has a really important role. Some breeds are thought to be more predisposed to this pathology such as: Yorkshire Terrier, Cocker, Dachshund. The clinical signs can range widely; many patients are subclinical (especially the ones with chronic pancreatitis), while others can have a variety of gastro-intestinal signs. We can divide this signs into characteristic ones (such as vomiting, diarrhea, apathy, abdominal pain) and uncharacteristic signs (such as ascites, jaundice, fever). Seeing the complexity of this pathology, just a simple clinical examination won't help us diagnose it, but will give us clues for what paraclinical exams to take. This article will present the correlation between ultrasound imagistic findings considered to be the main tool for examination of the pancreatic structure and canine pancreatic lipase (also known as CpL), considered to be the golden standard for detecting pancreatic disorders.

Key words: Pancreatitis, CpL, Ultrasound

Introduction

Pancreatitis represents the most often met pathology of the exocrine pancreas. It can be acute or chronic, depending of the type of tissue lesion. Both forms can be severe (Ettinger S., Feldman E., Cote E., 2016). The causes are still under research but there are some predispositions for dogs with an improper diet or nutritional deficiency. Severe traumas, some types of surgery or large number of triglycerides combined with hyperadrenocortisim (Cushing syndrom) have been also remarked (Xenoulis PG, Levinski MD, Suchodolski JS, et al., 2011). Some breeds can be predisposed for this affection such as: Schnauzer, Yorkshire Terrier, Cocker, Dachshund or Poodle (Bishop MA, Xenoulis PG, Levinski MD, et all, 2010; Furrow E, Armstrong PJ, Patterson EE., 2012)

In the initial phase, the pancreatic juice secretion reduces and some factors will make the pancreatic enzymes to activate inside the pancreas instead of the intestinal tract. The enzymes will auto-digest the pancreas, determining the inflammation and the lesions observed at the level of the parenchyma (Hess RS, Kass PH, Shofer FS, et al., 1999). The clinical sings have a wide range, from apathy to vomiting, dehydration, abdominal pain or diarrhea. Some cases can be asymptomatic or have unspecific signs (Cook AK, Breitschwerdt EB, Levine JF, et al, 1993).

The diagnosis of this pathology requires a good anamnesis, a clinical examination, blood tests and imagistic exams.

The main imagistic exam for pancreatic assessment is represented by ultrasonography. It has limitations depending on the equipment, operator experience or the severity of the pathology. In case of pancreatitis some changes have been observed at the level of echogenicity, echotexture, reaction of the surrounding mesentery or peripancreatic free fluid. This changes can also be used in a scoring system for assessing the severity of the disease (Cridge H, Sullivant A.M., Wills R., Lee A., 2020).

Canine pancreatic lipase is considered at the moment the golden standard for the diagnostic of pancreatitis in alive dogs. It is the only measurement that focuses only on the pancreatic lipase. Studies have shown that its sensitivity is under 100%, reaching 86% in some studies compared with pathologic findings (Trivedi S.,

Marks S.L., Kass P.H., et all, 2011) and for a better result it is required to have a good history of the patient, clinical signs and imagistic diagnosis (Cridge H, Sullivant A.M., Wills R., et all, 2020).

MATERIAL AND METHOD

In the period of 1st of January 2023 and 1st of October 2023 a total number of 390 male dogs were seen at the Faculty of Veterinary Medicine Iasi, in the Imagistic Department and at the Endoscopy and Minimally Invasive Surgery Center from Bucharest. All this dogs had a clinical examination and abdominal examination; a total of 51 dog from the 390 had pancreatic changes on ultrasonography and gastro-intestinal signs. From the 51 dogs only 37 had a canine pancreatic lipase measured.

The ultrasonography was made on a GE Logiq V5 (figure 1) machine in Iasi and on a Samsung HS50 machine (figure 2) in Bucharest. On both machines the same imagist evaluated the cases using a microconvex and linear probe. Lateral and dorso-ventral recumbency was used depending on the cooperation of the patient.

The canine pancreatic lipase (CpL) was measured quantitative in both cities with different devices.

The ultrasound changes were represented by 1 or more of the next: change in size, change in echogenicity, change in echotexture, surrounding mesentery reaction, peripancreatic free fluid, changes of the duodenum near the right pancreatic lobe (with 1 or more of the anterior changes) or pain during the deep palpation of the pancreatic area with the ultrasound probe.

The CpL was categorized in 3 stages: 0-200 µg/L (no pancreatitis), 200-400 µg/L (suspicion of pancreatitis and over 400 µg/L (pancreatitis).

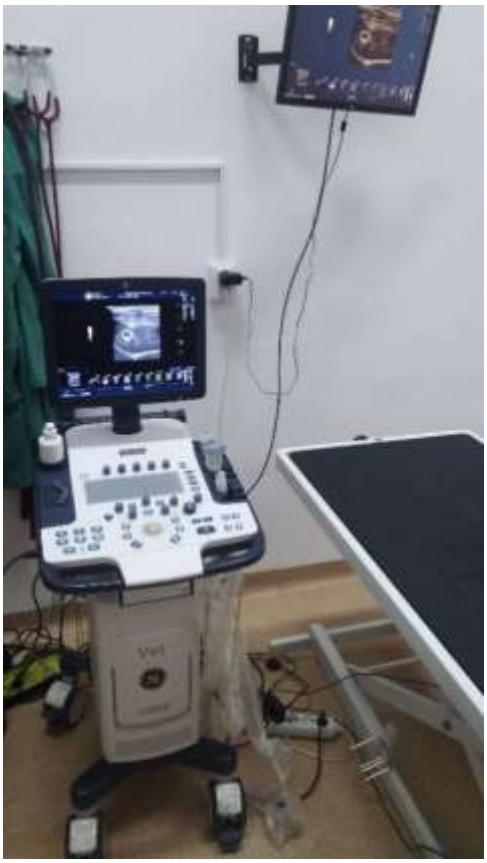

Figure 1. GE Logiq V5 ultrasound machine

Figure 2. Samsung HS 50 machine

RESULTS AND DISCUSSIONS

During the period 1st of January and 1st of October a number 390 cases were seen on ultrasonography, from these 51 had pancreatic lesions, meaning 13%. From the 51 cases, only 37 of them had the value of canine pancreatic lipase measured and are part of the study.

37 cases had a full clinical examination, ultrasound examination and canine pancreatic lipase measured from which 23 were males and 14 females. There is a larger number of males then females, without any clinical or pathological meaning.

Regarding age, patients were categorized in 2: under 7 years, a number of 8, where the average age was 4.6 years ranging from 2 to 7 years; over 7 years a number of 29, where the average age was 10.9 years ranging from 8 to 15 years. There is a 78.3% (29) of cases being over 7 years, an explanation would be the subclinical pathologies of the pancreas that appeared in time and left lesions at this level.

Most breeds were represented by small sizes such as Yorkshire terrier (a number of 13), Bichon (a number of 5), Beagle, French bulldog, Jack Russel, Italian whippet, Teckel but also a small number of medium sized such as Labrador (2), mixed breeds or Swiss shepherd. A reason for the large number of small size breed could be explained by the longevity (having more chances in life to have different subclinical or clinical lesions) and many of them living in an indoors lifestyle with a mixed diet (including same food ate by the owners).

Ultrasonographic changes were seen as change in size, change in echogenicity, change in echotexture, surrounding mesentery reaction, peripancreatic free fluid or pain during the deep palpation of the pancreatic area with the ultrasound probe. 13 of the patients had only one of this changes while the rest of 24 had 2 or more. The most seen changes were echogenicity (Figure 3) changes in 32 out of 37, size changes (Figure 3,4) in 16 out of 37, echotexture changes in 12 out of 37 (figure 5) and pain in 5 out of 37.

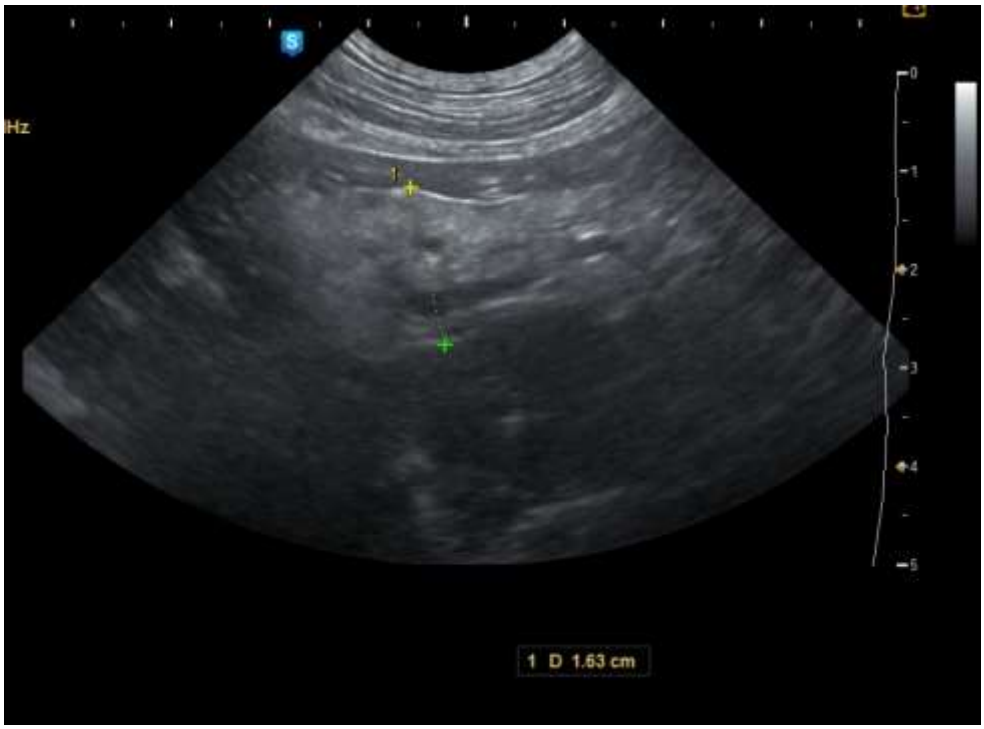

Figure 3. Hyperechoic pancreas with enlargement

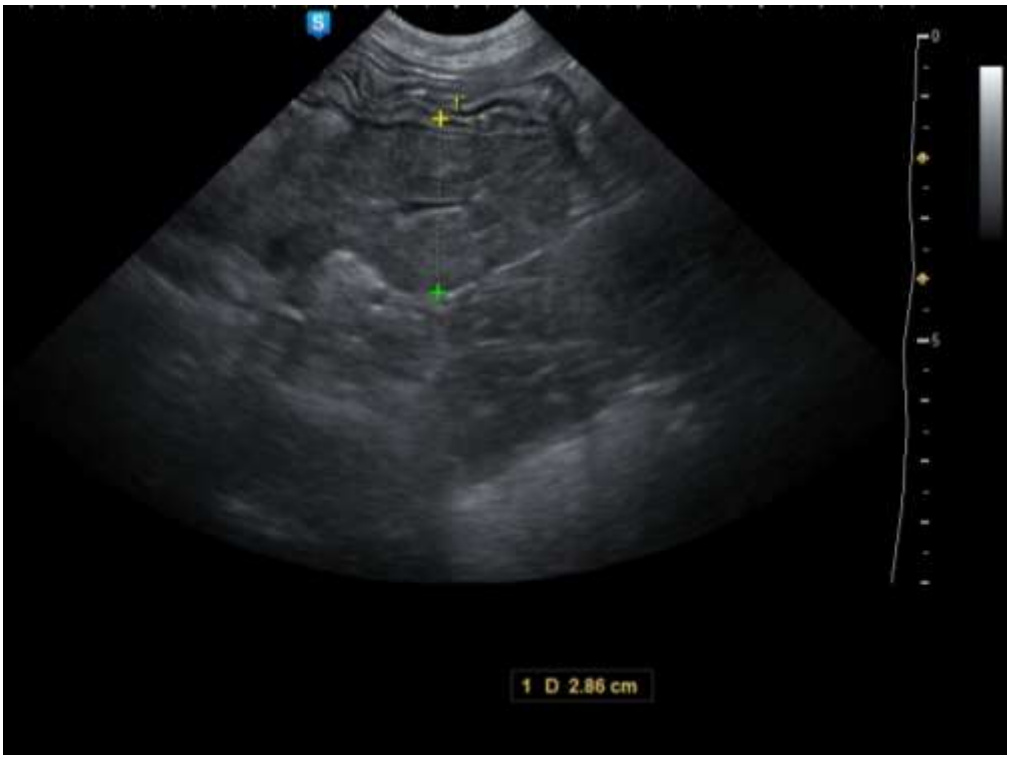

Figure 4. Enlarged pancreas with corrugated duodenum

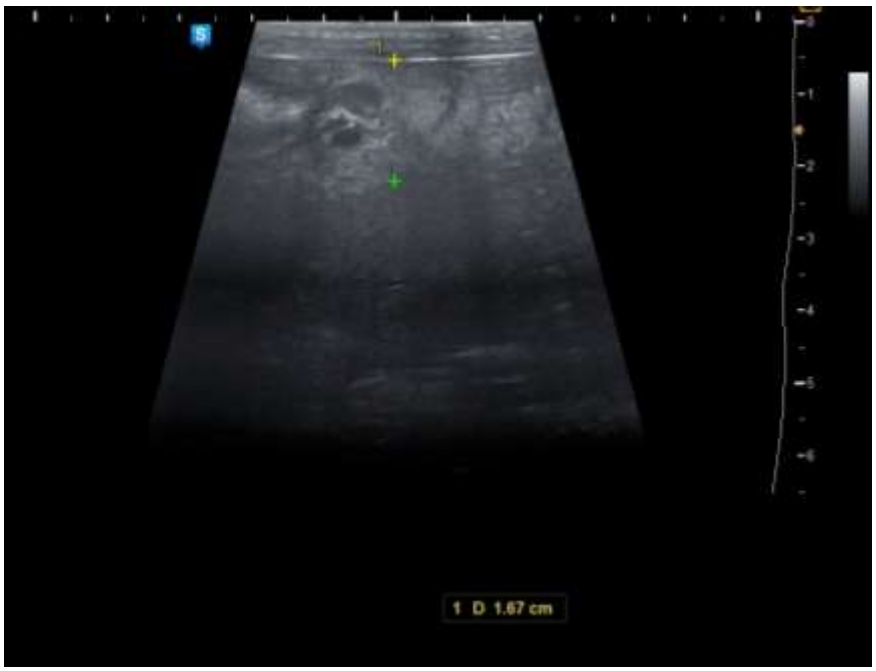

Figure 5. Enlarged pancreas with changed echotexture

The canine pancreatic lipase was categorized in 3 with the following results: bellow 200 µg/L with a number of 8 patients that had an average of 90.3 µg/L and a range between 10 and 158 µg/L; between 200 and 400 µg/L, with a number of 10 patients that had an average of 322.1 µg/L and a range between 221 and 395 µg/L.; over 400 µg/L, with a number of 19 patients that had an average of 700 µg/L with a range between 409 and 1400 µg/L. We could conclude from this that only 8 from 37 patients with ultrasonographic lesion and gastro-intestinal clinical signs had a normal CpL while the rest of 29 had at least the suspicion of pancreatitis. The correlation would be that 78% of cases from this study, with pancreatic changes on ultrasonography, presented changes on canine pancreatic lipase suggestive for pancreatitis.

CONCLUSIONS

Diagnosis of pancreatitis requires a complex protocol involving clinical examination, imagistic exams and blood analyses. Adult and geriatric pacients are more predisposed to this pathologies, one reason being the lesions at the level of the pancreas accumulated during the years. Ultrasonography combined with canine pancreatic lipase will give us a good correlation towards confirming a pancreatitis diagnosis.

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PROACTIVE SANITARY-VETERINARY MONITORING OF BEE FAMILIES INCLUDED IN A PROPHYLAXIS PROGRAM (ACTIVE BEEKEEPING SEASON 2023)

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Abstract

The purpose of this work is to monitor the state of bee health through morphoclinical and laboratory examination of bees on samples of live bees and honeycombs with brood for the prevention and control of diseases in bees in order to include them in a study on the impact of the non-ionizing electromagnetic radiations on bees. Samples were collected during the active season 2023, and morpho-clinically and laboratory examined according to OIE regulations from 9 private apiaries (PH, AG, TR, CL, VN, IS, DB, VL and IF) from which we collected 18 samples. The laboratory results revealed the existence of some diseases in 6 apiaries (66.67%), as follows: a unique evolution (suspected foulbrood disease in one apiary) (11.11%), five apiaries with mixed conditions (55,56%) (varroosis, nosemosis, chalkbrood, and suspected poisoning), and three apiaries were diagnosed as clinically healthy (33.33%). Studying the influence of non-ionizing radiation on bees has wider implications for ecology and the environment, as bees play a crucial role in pollination and maintaining ecosystems. The interaction between bees and electromagnetic radiation is a complex and multifactorial issue that may explain the diversity of conclusions in the available studies. Therefore, studies are needed in Romania to better understand the connection between non-ionizing electromagnetic radiation and the decline of bee populations (*Collony Colapse Disorder*).

Key words: bees, private apiaries, monitoring, health

INTRODUCTION

Bees, being among the most important natural pollinators, play a crucial role in maintaining balance in natural ecosystems and ensuring agricultural production. However, they are frequently affected by a number of diseases that can threaten the health and survival of bee colonies (Alleri M., *et al*, 2023; Pattazhy S., 2011; Seker S.S., *et al*, 2022).

One of the main factors currently contributing to the outbreak of bee diseases is the stress caused by changes in their habitat, the intensive use of pesticides, malnutrition, electromagnetic radiation and other environmental factors that favor a decrease in immunity and the onset of various diseases (bacterial, fungal, viral, parasitical), including the colonies collapse disorder (Balmori A., 2014; Goudeseune L., *et al*, 2018; Treder M., *et al*, 2023; Watson K., Stallins J.A. 2016). Decreased pollination of wild plants can lead to increased population of plants that do not rely on pollinators, and decreased pollinators would lead to drastic declines in crop plants (Friesen M., Havas M., 2019; Gagnaire B., *et al*, 2019; Gawas A.U., 2015; Seker S.S., Simsek O., Brief 2022).

The concern about the impact of electromagnetic radiation on bees is part of a wider issue related to the potential impact of human activities on the environment and wildlife (Balmori A., 2015; Balmori A., 2021; Cammaerts M.C., Johansson O., 2014; Chiaraviglio L., *et al*, 2018; Cucurachi, S., *et al*, 2013; Dalio J.S., 2015; Formicki, K., *et al*, 2021; Friesen M., Havas M., 2019; Gagnaire B., *et al*, 2019; Gould J.L., 1989). This issue has gained increased attention in recent years as the implementation of new technologies such as 4G and 5G networks has led to increased exposure to electromagnetic radiation in many areas (Cucurachi S., *et al*, 2013; Field E., 2021; Goudeseune L., *et al*, 2018; Hasan H.J., et al, 2021; Jeladze V., *et al*, 2022; Korolev I.V., *et al*, 2022; Korolev I.V., *et al*, 2022; Seker S.S. *et al*, 2022).

There is no national level information on the current situation regarding the impact of electromagnetic radiation on bees in Romania, and therefore it is important to conduct research and monitor the impact of different sources of electromagnetic radiation, including 4G and 5G networks on bees and other pollinators, as they

play an important role in the ecosystem and in food production.

It is recognized that bees are important pollinators and play a critical role in maintaining biodiversity and food security. In recent years there has been increasing concern about the potential impact of various environmental stressors, including electromagnetic radiation, on bee populations and their health.

While some studies have suggested that exposure to electromagnetic radiation can have negative effects on bees (http://stop5gromania.ro/studii-ale-efector-radiatiilor-campurilor-electromagnetice-asupra-omului-animalelor-plantelor/; www. apc-romania.ro/ro/i-de-ce-ne-mor-albinele.htm; www.ncbi.nlm.nih.gov; www.youtube.com/ X5IhKHGDKhM), further research is needed to fully understand the extent of these impacts and to develop appropriate strategies to mitigate them or protect bee colonies through various screening devices.

In Romania, as in other countries, it is important to assess the potential impacts of electromagnetic radiation on bee populations and to implement measures to protect them. This may include monitoring bee populations, monitoring exposure to electromagnetic radiation, and conducting research to better understand the effects of these radiation sources on bee health (Favre D., Johansson O., 2020; Favre, D. 2017; Thielens A., *et al*., 2020).

The situation regarding the impact of electromagnetic radiation on bees is still a subject of ongoing research and debate in Europe and in the world. While some studies have suggested that exposure to electromagnetic radiation can have negative effects on bees, including changes in their behavior, flight and communication, the results of these studies have been unclear and are not yet definitive.

Some studies have suggested that exposure to electromagnetic radiation from sources such as cell phone towers (Odemer R., Odemer F., 2019; Panagopoulos D.J., 2019; Panagopoulos D.J., *et al.*, 2015) and Wi-Fi networks can have negative effects on bees, including changes in behavior and their ability to navigate (Bozorgmanesh M.A., Kowkabi F., 2019; Bozorgmanesh M.A., Kowkabi F., 2023; Favre D., 2011; Kumar N.R., *et al*, 2011; Kumar R., *et al*, 2021; Kumar, S.S., 2018; Seker S., *et a*l, 2022; www.apc-romania.ro; www.ncbi.nlm), other studies suggest that these effects are negligible or within normal limits for bees or find no significant impact on them.

Electromagnetic radiation comes from a wide range of sources (mobile communications networks, Wi-Fi, power lines and other electronic devices) that can affect the behavior, orientation and communication of bees, as they use orientation information in the electromagnetic spectrum to navigate and find food and hives (Abdelaal A., 2015; Balmori A., 2015; Balmori A., 2021; Greggers, U., *et al*, 2013; Hsu C.Y., *et al*, 2007). Exposure to radiation emitted by mobile phones can affect bees' ability to find and return to their own hive, negatively impacting colony activity and health (Bozorgmanesh M.A., Kowkabi F., 2019; Bozorgmanesh M.A., Kowkabi F., 2023; Favre D., 2011; Gawas, A.U., 2015; Gould, J.L., *et al*, 1989; Kumar N.R., Sangwan S., Badotra P., 2011; Kumar R., *et al.*, 2021; Kumar S.S., 2018; Sharma V. P., Kumar N. R., 2010; Treder M., *et al*, 2023; Watson K., Stallins J. A. 2016).

Overall, the situation regarding the impact of electromagnetic radiation on bees is complex and is an area that requires continuous research and monitoring. It is also important to ensure that these networks are used in a safe and responsible way and to assess the potential risks and impacts on human health and the environment (Adlienė D., *et al*, 2020).

Further research and monitoring is needed to fully understand the potential effects and to determine the best approach to mitigate any negative impact on bee populations and to inform policy makers in decision-making in Romania and other countries. It is also important for policy makers to consider the potential impacts of electromagnetic radiation when developing and implementing policies related to 4G and 5G networks and other sources of electromagnetic radiation, and to implement measures to protect bee populations and other important pollinators.

Despite ongoing research, there is still no clear consensus on the impact of electromagnetic radiation on pollinators. Currently, the scientific consensus is that more research is needed to fully understand the potential impact of electromagnetic radiation on pollinators. Some countries, including the European Union, have taken steps to monitor the development of 5G technology and its potential effects on the environment and pollinators, and have implemented regulations to limit EMF exposure in certain areas, such as around beehives and feeding grounds for bees to minimize potential risks to pollinators and other wildlife.

MATERIAL AND METHOD

The samples were collected in the active beekeeping season 2023, morphoclinically and laboratory examined according to OIE regulations (2008) and adapted in the Pathology Laboratory of

the SCSBB. We investigated collected samples from the experimental group consisting of none private apiaries (PH, TR, AG, IF, CL, VN, VL, IS and DB) (Figure 1) having a total of 412 bee families (*table 1*), from which 18 samples were collected (9 live bees' samples and 9 brood combs samples corresponding to each apiary) (*table 2*).

Figure 1. The locations where the monitored bee colonies are located (Experimental Lot)

Table 1.
Bee families from the private apiaries studied

EXPERIMENTAL LOT	NO. BEE FAMILIES
Apiary 1 (PH)	60
Apiary 2 (TR)	85
Apiary 3 (AG)	85
Apiary 4 (IF)	25
Apiary 5 (CL)	12
Apiary 6 (VN)	40
Apiary 7 (VL)	20
Apiary 8 (IS)	35
Apiary 9 (DB)	50
TOTAL	412

Table 2.
Number of samples of bees and brood combs collected from the private apiaries

EXPERIMENTAL LOT	Bee samples/apiary	Brood combs samples/apiary
Apiary 1 (PH)	1	1
Apiary 2 (TR)	1	1
Apiary 3 (AG)	1	1
Apiary 4 (IF)	1	1
Apiary 5 (CL)	1	1
Apiary 6 (VN)	1	1
Apiary 7 (VL)	1	1
Apiary 8 (IS)	1	1
Apiary 9 (DB)	1	1
TOTAL	18	

RESULTS AND DISCUSSIONS

The laboratory results revealed the existence of diseases in 6 private beehives (66.67%), as follows: a single disease (varroosis in one apiary) (11.11%), 5 apiaries with mixed diseases (varroosis, nosemosis, chalkbrood/petrified brood, suspicion of intoxication) (55.56%), and 3 apiaries were diagnosed as clinically healthy (33.33%) (*table 3, figures 2, 3, 4, 5, 6 and 7*).

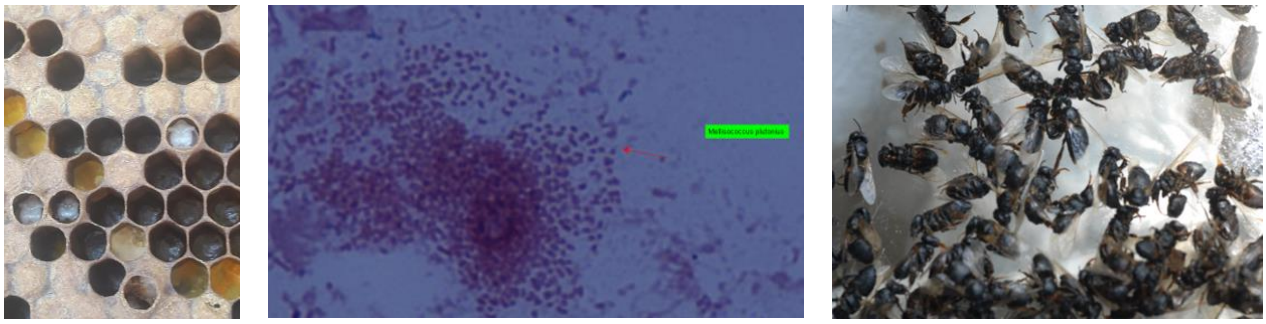

Figure 2. Mixed disease diagnosis (suspected European foulbrood and intoxication) to private apiary 1 (PH)

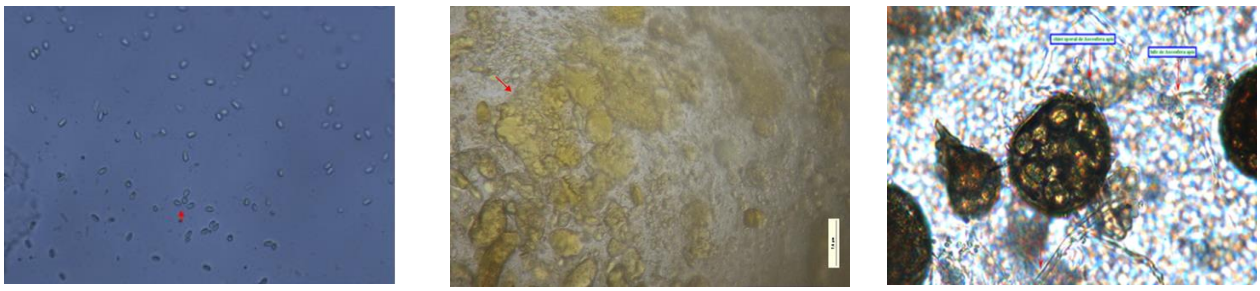

Figure 3. Mixed disease diagnosis (nosemosis + chalkbrood) to private apiary 2 (TR-1), Ascospheres with ascospores and hyphae of A. apis from the gut of a live bees (directly prepared, x 400)

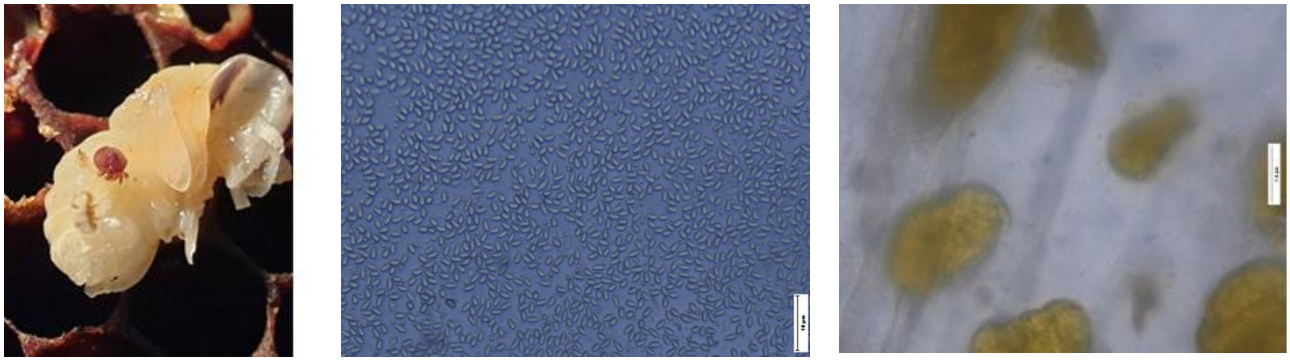

Figure 4. Mixed disease diagnosis (varroosis + nosemosis + chalkbrood) to private apiary 5 (CL)

Figure 5. Mixed disease diagnosis (varroosis + chalkbrood/petrified brood) to private apiary 7 (DB)

Figure 6. Diagnosis of varroosis (single condition) to private apiary 8 (IS)

Figure 7. Mixed disease diagnosis (varroosis + chalkbrood/petrified brood) to private apiary 9 (DB)

Table 3.
Diseases diagnosed in the studied private apiaries (Experimental Lot)

Private apiaries	Disease	Diagnosis
Apiary 1 (PH)	mixed	suspicion of European foulbrood and intoxication
Apiary 2 (TR-1)	mixed	nosemosis + chalkbrood
Apiary 3 (AG)		clinically healthy
Apiary 4 (IF)		clinically healthy
Apiary 5 (CL)	mixed	varroosis + nosemosis + chalkbrood
Apiary 6 (VN)		clinically healthy
Apiary 7 (VL)	mixed	varroosis + chalkbrood/petrified brood single varroosis
Apiary 8 (IS)	single	varroosis
Apiary 9 (DB)	mixed	varroosis + chalkbrood

CONCLUSIONS

The morpho-clinical and laboratory examination on live bees and brood comb, carried out in 2023 on 9 private apiaries (which will be included in an experimental study on the impact of non-ionizing electromagnetic radiation on bees), established the following: bee families from 3 apiaries were found clinically healthy (apiary no. 3, 4 and 6) (33.33%); diagnosis of a disease with a unique evolution in a apiary - varroosis (apiary no. 8), and mixed disease diagnosis in 5 apiaries (apiary no. 1, 2, 5, 7 and 9).

Studying the influence of non-ionizing radiation on clinically healthy and disease-affected bees has wider implications for ecology and the environment, as bees play a crucial role in pollination and ecosystem maintenance.

The interaction between bees and electromagnetic radiation is a complex and multifactorial issue, with numerous variables (radiation intensity, frequency and duration of exposure, types of radiation, health status, etc.) that may explain the diversity of conclusions from the available studies.

Therefore, studies are needed in Romania to better understand the connection between non-ionizing electromagnetic radiation and the decline of bee populations (*Colony Collapse Disorder*).

Compliance with ethical standards: The research does not involve human and/or animal experimentation.

Conflict of interest: The authors declare that they have no conflict of interest. We mention that the research conducted has no connection with the activity of official territorial or central laboratories nominated for the monitoring and control of bee diseases.

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w**ww.apc-romania**.ro/ro/i-de-ce-ne-mor-albinele/NjI5LTE.html

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www.youtube.com/watch?v=X5lhKHGDKhM Dovezi ale nocivității extreme a implementării 5G **(**milioane de albine mor în zona antenelor 5G).

DETECTION OF BETA-LACTAM RESIDUES IN ENVIRONMENTAL AND DRINKING WATER BY IMMUNOENZYMATIC ASSAY

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Abstract

In recent decades, concern over emerging organic contaminants in the environment has grown considerably because of their potentially harmful effects on organisms and ecosystems. These synthetic compounds are widely used in modern life-style and due to improvements in analytical technologies, we are now able to identify and quantify them even in small concentrations. One of the most important pharmaceutical contaminants is antibiotics, of which more than half belong to the class of beta-lactams. This research aimed to determine the beta-lactam antibiotics residues in surface water (rivers) and groundwater, which serve as public or private sources of drinking water, as well as in urban wastewater. The samples were collected from different places throughout the Western part of Romania and analyzed using commercially available ELISA kits for the detection of beta-lactams in liquid samples. The results show that beta-lactam antibiotics are ubiquitous in all categories of water and establish the ELISA method as an acceptable screening tool for antibiotic residues.

Key words: antibiotic residues, drinking water, ELISA

For a long time, the impact of chemical pollution on the environment, human and animal health, has been based on monitoring priority pollutants and in particular those classified as persistent, bioaccumulative and toxic, known as "persistent organic pollutants" (POPs). The inclusion of these compounds in the list of priority hazardous substances was made not only as a result of risk identification and analysis but mainly because they could be identified in the environment at very low concentrations, by the analytical techniques available at the time (Daughton C.G. *et al*, 2001).

The improvement of analytical techniques in recent years has made it possible to detect even small quantities of natural or synthetic substances in the environment, which are usually not monitored and pose unknown risks to human health and ecosystems. These substances are termed "emerging organic contaminants" (EOCs) (Richardson S.D. *et al*, 2011; Stuart M. *et al*, 2012). EOCs are not necessarily newly discovered substances, but rather compounds that have long been reaching the environment (water, air, soil) via various pathways, but whose consequences are only now becoming known. The class of emerging pollutants includes compounds from industry, disinfectants, detergents, pharmaceuticals, and personal care products (PPCPs), as well as lifestyle substances such as caffeine and nicotine (life-style compounds). The EOCs list remains open and as analytical detection methods are refined, new substances will be included (Meffe R. *et al*, 2014).

The PPCPs category includes also antibiotics, pharmacological agents widely used in both human and veterinary medicine. After administration, they are excreted 5% to 90% unmetabolized or as active or inactive metabolites, but may subsequently be converted back to their original compounds (Dinh Q.T. *et al*, 2011; Mirzaei R. *et al*, 2018; Sarmah A.K. *et al*, 2006; Yan Q. *et al*, 2014). Thus, a significant amount of antibiotic residues ends up in urban wastewater, through sewage treatment plant effluents, in biofertilizers used in agriculture and from here, in surface and groundwater (Lapworth D.J. *et al*, 2012).

Wastewater treatment removes biodegradable organic compounds, nitrogen, phosphorus and in certain amounts pathogens, but the technology is not designed to retain the micro-organic pollutants. Thus, antibiotic residues cannot be removed efficiently and in some cases, they have been identified in higher concentration in the

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effluent than in the influent (Yan Q. *et al*, 2014; Gao L. *et al*, 2012). The increased interest of researchers in the problem of antibiotic residues in the environment has led to the identification of these compounds in all water categories: surface water (Dinh Q.T. *et al*, 2011; Feitosa-Felizzola J. *et al*, 2009), groundwater (Barnes K.K. *et al*, 2008; Wolf L. *et al*, 2012) and drinking water (Charuaud L. *et al*, 2019; Focazio M.J. *et al*, 2008; Padhye L.P. *et al*, 2014). Antibiotics are considered "pseudo-persistent" pollutants, as their persistence in the environment is due to their continuous intrusion (Huang C.H. *et al*, 2001).

The beta-lactam class includes penicillins and their derivatives, cephalosporins, carbapenems, monobactams and beta-lactamase inhibitors (Ungureanu V.A., 2018). Although in recent years, there have been attempts to identify residues of beta-lactam antibiotics in aquatic environments: wastewater, surface waters (Bruno F. *et al*, 2001; Cha J.M. *et al*, 2006; Christian T. *et al*, 2003) and groundwater (Sacher F. *et al*, 2001), many of the targeted compounds could not be detected or were found in much lower concentrations than antibiotics belonging to other antimicrobial classes. This is explained by the instability of the beta-lactam ring, which can be easily disintegrated by bacterial beta-lactamases and by chemical reactions occurring in aquatic environments (hydrolysis, photolysis) (Christian T. *et al*, 2003). Some of the degradation compounds resulting from such reactions are more stable and thus easier to detect, which is why monitoring programs may consider determining them instead of identifying the parent structures (Sy N.V. *et al*, 2017). On the other hand, as in the case of cephalosporins, the products resulting from their photodegradation in aquatic environments are more stable but also more toxic compared to the parent compounds (Wang X-H *et al*, 2012).

The danger of antibiotic residues in drinking water is primarily due to the extent of antibiotic resistance that has developed in recent years. According to the World Health Organisation, this phenomenon is one of the greatest threats to public health (WHO, 2018). In the environment (soil, water), antibiotic residues are detected in concentrations of nanograms or micrograms per volume (liter) or mass (kilogram) (Hon N.T.N. *et al*, 2016). Although such concentrations do not inhibit bacterial multiplication, some studies claim that they can cause the selection of resistant microorganisms, in particular through mechanisms involving mutations in bacterial DNA, thus contributing to the transformation of aquatic environments into reservoirs of resistant germs (Kim S. *et al*, 2012).

On the other hand, in the case of beta-lactam antibiotics, there is a risk of allergic phenomena in sensitive individuals (Cha J.M. *et al*, 2006). No less important is the impact that these substances have on the diversity and functions of microorganisms in aquatic ecosystems, most frequently leading to the selection of resistant populations and the extinction of less tolerant ones (Rosi E.J. *et al*, 2018).

For the quantification of antibiotic traces in the environment (water, soil), animal waste, or animal products, the most commonly used analytical method is the high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) (LC/MS/MS). Although the advantages of this technique in detecting extremely low concentrations of residues have consecrated it as the gold-standard method, its high costs and the impossibility of processing a large number of samples simultaneously make it less useful in the screening process (Aga D.S. *et al*, 2016; Zhang Z. *et al*, 2013).

The immunoenzymatic technique (ELISA), on the other hand, has demonstrated its usefulness both as a screening tool and in the quantitative assessment of residues. Even though assay kits are generally designed for the determination of a single substance, the antibodies used show specificity against the whole class of antibiotics, cross-reacting with all structurally similar compounds. While even small changes in the structure of the analyte make it impossible to identify it by LC/MS/MS, the cross-reactivity of ELISA is an advantage in screening processes, as it also allows the detection of metabolites of the parent substance. Thus, cross-reactivity also becomes an advantage when measuring the bioavailability of the degradation products of a particular antibiotic, especially if they also exhibit biological activity (Aga D.S. *et al*, 2016).

A number of studies have relied on the enzyme-linked immunosorbent assay method in the detection of antibiotic traces in soil and animal excreta (Aga D.S. *et al*, 2003), groundwater (Barber L.B. *et al*, 2009; Bradley P.M. *et al*, 2014), surface water (Kumar K. *et al*, 2004) and wastewater (Černoch I. *et al*, 2012). Others have aimed to identify products resulting from the degradation of antibiotics in aquatic environments and concluded that ELISA is superior to HPLC for such determinations (Hu D. *et al*, 2008). Even if ELISA cannot fully replace the gold-standard quantitative analytical methods, there are strong arguments for its use as a screening tool due to: low cost, short working time, the possibility to test a large number of samples simultaneously, and above all, due to cross-reactivity, which also

allows the detection of metabolites that have entered the environment (Aga D.S. *et al*, 2016).

The aim of the study was to determine the residues of beta-lactam antibiotics in surface water (rivers) and groundwater, the last serving as public or private drinking water sources, or being used for watering animals or irrigating vegetable crops, as well as in urban wastewater. The samples were collected from different places throughout the Western part of Romania and analyzed using commercially available ELISA kits for the detection of beta-lactams in liquid samples.

MATERIAL AND METHOD

A total of 42 water samples from various sources were collected, processed, and analyzed in two stages. The first stage of sampling (S I) took place during a period of dry weather conditions. The second stage (S II) took place during a period in which surface and groundwater flow was increased due to abundant rainfall.

Surface water samples were collected from a drainage channel that flows into the Bega Veche River in Timiș county and from the Bistra River in Caraș-Severin county, Timiș River in Caraș-Severin and Timiș counties, Bega river in Timiș county and Mureș river in Arad county.

Wastewater samples were taken from the influents and effluents of four water treatment plants located in Timiș county, more precisely Timișoara, Jimbolia, Dudeștii Noi, and Lugoj (effluent only). The effluents of all four plants are discharged into natural water bodies.

Drinking water intended for human consumption was sampled from groundwater sources. The samples were taken from artesian wells in Chizătău, Dudeștii Noi, Ohaba-Forgaci, and Sinersig, from citizens' household wells in Ciuta and Țela, from the public water distribution network (groundwater) in Dudeștii Noi, Jimbolia, Lugoj, and Ohaba-Forgaci, as well as from public wells and the public distribution network (surface water) in Lugoj and Timișoara.

Further samples were taken from two shallow drilled wells (maximum 10 meters) for the watering animals and gardens, and from a source of natural mineral water intended for human consumption at the Buziaș spa resort in Timiș county. The waters of Buziaș are characterized by a high content of minerals, especially iron, as well as a high concentration of carbon dioxide.

The water samples were collected in clean glass containers with a capacity of 750 ml. River water was collected from bridges with the help of a bathometer. The samples were kept overnight at 4°C and analyzed in the following morning.

Max-Signal® Beta-Lactam ELISA kits (PerkinElmer Inc.), which are based on a competitive enzyme immunoassay for the quantitative analysis of beta-lactams were used for the analysis of the water samples. They allow the detection of beta-lactams such as ampicillin, penicillin G, amoxicillin, cloxacillin, and ceftiofur in milk, meat, egg, urine, serum, plasma, and a variety of other samples.

Each well on the plate contained in the ELISA kits is lined with the pharmaceutical substance of interest, in this case, beta-lactam antibiotics (reference antigens). The test samples were distributed into the wells and a protein with a high affinity for beta-lactams (primary antibodies, not enzyme-conjugated) was added. If there are beta-lactam residues in the sample, they will bind to the protein, not allowing it to bind to the reference antigens in the wells. Once all of the residues have been attached to the proteins, the excess protein binds to the reference antigens. A solution with enzyme-conjugated detection antibodies (secondary antibodies, peroxidase) is then added. These antibodies attach to the protein that is bound by the control antigen in the wells. 3,3',5,5'-tetramethylbenzidine (TMB) is added in the last step to obtain the color reaction that can be read in a spectrophotometer as absorbance values. The intensity of the color reaction is inversely proportional to the concentration of beta-lactam residue in the sample.

The limit of detection for milk is 0.4 ng/ml and the limit of quantification is 0.8 ng/ml. They were obtained by multiplying the limits of detection and quantification of the test, 0.04 ng/ml and 0.08 ng/ml, respectively, with the dilution factor for milk, which is at least 10.

The water samples were processed according to the test kit manufacturer's instructions, but they were not diluted. Without a dilution factor, we defined the limit of quantification for water as 0.08 ng/ml. The device's software also provided results that were lower than 0.08 ng/ml, as the limit of detection is 0.04 ng/ml, however, these findings lie outside of the measurement range and were therefore considered insignificant.

The optical density was analyzed spectrophotometrically at 450 nm. The concentration of beta-lactams in the sample was determined by comparing the color intensity with the standard curve (log-logit), which was established using the concentration (x-axis)

relative to the absorbance (y-axis) of each standard provided by the ELISA kit: negative control standard 0 ng/ml, standard 0.08 ng/ml, standard 0.2 ng/ml, standard 0.4 ng/ml, standard 0.8 ng/ml, and standard 1.2 ng/ml. Since the determinations were made in two stages, a standard curve was established for each stage (figure 1).

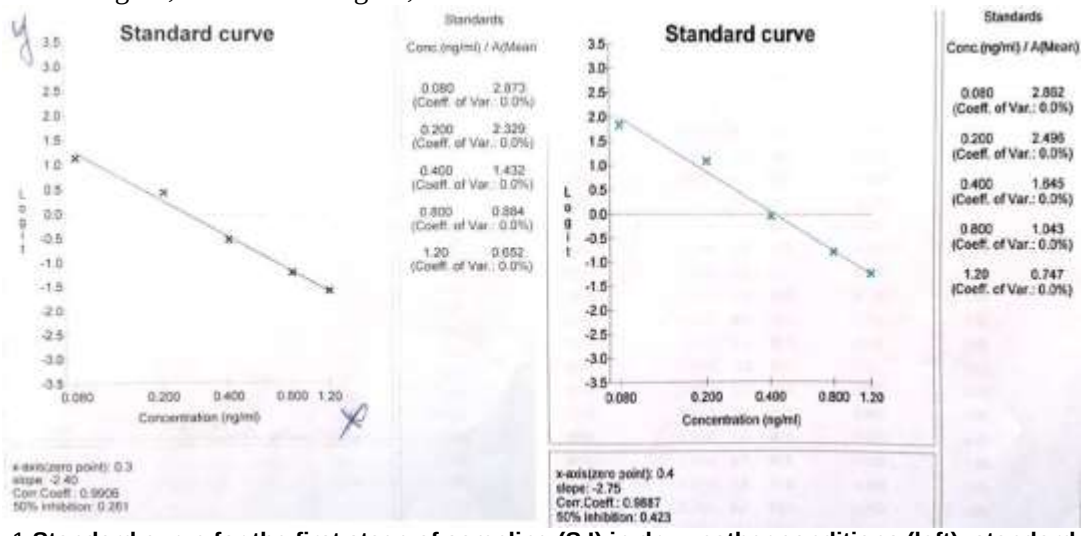

Figure 1 **Standard curve for the first stage of sampling (S I) in dry weather conditions (left); standard curve for the second stage of sampling (S II) in rainy weather conditions (right).**

The absorbance was expressed in percentage (B/B0) and calculated according to the formula:

Relative absorbance % = (standard absorbance of the sample / standard absorbance of the negative control)×100

Characteristics of the ELISA test kit that were taken into consideration were the high recovery rate of over 80% and the intra-assay and inter-assay coefficient of variation (normally below 8%). According to the data provided by the manufacturer, the test specificity is 100% for penicillin G, >100% for ampicillin, cefoperazone, ceftiofur, cloxacillin, cloxacillin, nafcillin, and oxacillin, and 44% for amoxicillin.

The removal efficiency of wastewater treatment for beta-lactam antibiotic residues was calculated according to the formula:

Removal efficiency % = [(influent value - effluent value) / influent value]×100 (Bradley P.M. et al, 2014).

RESULTS AND DISCUSSIONS

Out of the total samples that were analyzed (42), 59.52% (25) had values above the minimum limit of quantification (0.08 ng/ml). None of the samples were below the limit of detection (0.04 ng/ml). The remaining samples (17) had values between 0.055 and 0.079 ng/ml. The highest number of quantifiable samples was obtained for wastewater, followed by surface water. The lowest number was found for groundwater (*table 1*), as expected. Vulliet *et al.* (2011) analyzed surface and groundwater intended for human consumption for residues of pharmaceutical and hormonal substances and found that the detection frequency and concentrations of the target compounds are higher in surface waters. The only substance that was more frequently and in higher concentrations detected in groundwater was sulfamethoxazole (Vulliet E. *et al*, 2011).

Studies pertaining to the detection of beta-lactam antibiotic residues in the environment using enzyme-linked immunosorbent assays are few. Most of the research utilizing the ELISA method for surface water (Kumar K. *et al*, 2004), wastewater (Černoch I. *et al*, 2012), and groundwater (Barber L.B. *et al*, 2009; Bradley P.M. *et al*, 2014) analysis was done to identify substances from the class of sulfonamides (Barber L.B. *et al*, 2009; Bradley P.M. *et al*, 2014; Černoch I. *et al*, 2012). Sulfonamides are the most commonly found compounds in aquatic environments. They are found in concentrations ranging from 10 μg/L to 1 mg/L and unlike beta-lactams, which are unstable and rapidly degraded by hydrolysis or photolysis, sulfonamides are much more stable or even non-degradable (Cha J. *et al*, 2018). Recently, Ecke and Schneider (2021) presented the first example of utilizing hydrolysis of beta-lactam antibiotics for the improved immunochemical determination of these substances and their hydrolysis products and provided a method for the quick evaluation of the contamination of drinking water with pharmaceutical compounds and therefore the risk

of potential microbiological resistance development (Ecke A. *et al*, 2021).

The percentage of quantifiable samples of surface water was 69.23% (9). The values were between 0.087 ng/ml for water collected from the Bega River in the center of Timișoara in the first stage of sampling (S I) and 0.115 ng/ml for the Bistra River in the second stage of sampling (S II), which was characterized by abundant rainfall and increased river flow. Four of the 13 surface water samples had values close to the limit of quantification, between 0.072 and 0.079 ng/ml (*table 1*). Using the HPLC technique, Christian et al. (2003) found traces of amoxicillin, ampicillin, flucloxacillin, mezlocillin, and piperacillin in surface waters in concentrations of no more than 0.048 ng/ml. On average, beta-lactams were found in concentrations of less than 0.01 ng/ml (Christian T. *et al*, 2003). Using the same analytical method, Cha et al. (2006) found values of beta-lactam residues in surface water between 0.009 and 0.011 ng/ml (Cha J.M. *et al*, 2006).

Table 1

Levels of β-lactams in aqueous environmental samples

Water category and location				Concentration ng/ml		Samples with quantifiable values
				S I	S II	
Surface water (13)	Rivers	Bistra	Ciuta		0.115	**9 (69.23%)**
		Timiș	Downstream Caransebeș		0.105	
			Upstream Lugoj		0.102	
			Upstrea Lugoj drinking water treatment plant II	0.079*		
			Influent of the Lugoj drinking water treatment plant II		0.089	
		Mureș	Săvârșin		0.075*	
		Bega	Lipova		0.098	
			Făget		0.099	
			Bucovăț		0.097	
			Influent of the Timisoara drinking water treatment plant	0.093		
			Center of Timișoara	0.087		
	Drainage channel	Upstream Dudeștii Noi wastewater treatment plant		0.076*		
		Downstream Dudeștii Noi wastewater treatment plant		0.072*		
Wastewater (7)	Timișoara	Influent			0.528	**5 (71.42%)**
		Effluent			0.131	
	Jimbolia	Influent			0.471	
		Effluent			0.148	
	Dudeștii Noi	Influent		0.072*		
		Effluent		0.055*		
	Lugoj	Effluent		0.094		
Drinking water (22)	Public distribution network (surface water)	Lugoj (Timiș)		0.078*		**11 (50%)**
		Timișoara (Bega)			0.276	
	Public distribution network (groundwater)	Ohaba-Forgaci		0.082		
		Dudeștii Noi		0.075*		
		Lugoj water treatment plant I		0.069*		
		Lugoj water treatment plant III		0.074*		
		Jimbolia			0.153	
	Artesian wells/Springs	Ohaba-Forgaci		0.079*		
		Dudeștii Noi 1		0.067*		
		Dudeștii Noi 2		0.070*		
		Chizătău			0.079*	
		Sinersig			0.112	
		Buziaș			0.196	
	Public wells (groundwater)	Lugoj 1		0.078*		
		Lugoj 2		0.064*		
		Timișoara			0.085	
	Private wells (groundwater)	Ciuta 1			0.138	
		Ciuta 2			0.132	
		Țela 1			0.090	
		Țela 2			0.087	

Shallow drilled wells	Ohaba-Forgaci	0.082		
	Dudeștii Noi	0.075*		
Total no of samples		**20**	**22**	**42**
Unquantifiable values *		**15**	**2**	**17 (40.47%)**
Quantifiable values		**5**	**20**	**25 (59.52%)**

In a recent study that examined the presence of pesticides and veterinary medicines in 29 small waterways across 10 countries in the European Union, sulfonamide antimicrobials were identified in 48% of the samples, while the beta-lactams dicloxacillin and cloxacillin were found in 66% and 41% of the samples (Casado J. *et al*, 2019). These values reported by Casado et al. (2019) were detected by using the HPLC method and are similar to the percentage for beta-lactams in surface water identified by us, which validates the enzyme-linked immunosorbent assay as a much cheaper screening tool alternative compared to high-performance liquid chromatography (Aga D.S. *et al*, 2016; Zhang Z. *et al*, 2013).

Of the seven wastewater samples, 71.42% (5) were quantifiable. The samples that had values below the limit of quantification were those collected from the wastewater treatment plant serving the locality of Dudeștii Noi: 0.072 ng/ml in the influent and 0.055 ng/ml in the effluent. The data from *Table 1* suggests that the residue of beta-lactam antibiotics is reduced after the treatment process since the values of the effluent are lower than the values of the influent for all wastewater treatment plants (WWTPs) that were studied. The highest value in the influent was found for the wastewater treatment plant in Timișoara (0.528 ng/ml) and the highest value in the effluent was found for the wastewater treatment plant in Jimbolia (0.148 ng/ml). In the effluent of the wastewater treatment plant in Lugoj, 0.094 ng/ml of beta-lactam residues were found. For the wastewater treatment plant in Dudeștii Noi, the values for both influent and effluent were above the limit of detection, but below the limit of quantification. The removal efficiency of the WWTPs in this study was 75.18% for Timișoara, 68.57% for Jimbolia, and 23.61% for Dudeștii Noi. Using the HPLC method, Cha et al. (2006) found beta-lactam residues in the WWTP influents at values between 0.015 and 0.017 ng/ml. In the effluent samples, they were not detectable (Cha J.M. *et al*, 2006).

The two drinking water samples from public distribution networks (surface water) were collected in the cities of Lugoj and Timișoara. While the value for the sample from Lugoj was below the limit of quantification, the value for the sample from Timișoara was 0.276 ng/ml, which is the highest value we recorded excluding those of the WWTP influent samples (*table 1*).

Of the 20 drinking water samples from groundwater sources, 50% (10) contained quantifiable values of beta-lactam residue. The highest of these samples was found in the natural mineral water source at the Buziaș spa resort with a concentration of 0.196 ng/ml, followed by 0.153 ng/ml in water from the public distribution network in Jimbolia, which is treated with ozone. The rest of the quantifiable values were between 0.082 ng/ml from the shallow drilled well in Ohaba-Forgaci (Timiș county) and 0.138 ng/ml from one of the private wells in Ciuta (Caraș Severin county) (*table 1*). In one of the very few studies conducted in Romania on this topic, Szekeres et al. (2018) analyzed six groundwater sources with the HPLC technique and identified residues of cefepime and piperacillin with concentrations of 0.917 and 0.571 ng/ml, respectively, in two of the sources. Both of these sources were associated with animal farms in their vicinity. However, except for these two values, the overall distribution of beta-lactam antibiotic residues was low (Szekeres E. *et al*, 2018).

In Italy, Perret et al. (2006) identified traces of sulfonamides in sources of natural mineral water, from which it is collected, bottled, and marketed without any treatment (Perret D. *et al*, 2006). This is also the case with the natural mineral water at the Buziaș spa resort. The values obtained for the sample from this locality can be attributed to the presence of a recovery center, where significant quantities of pharmaceuticals are used.

For a more comprehensible presentation, the results from the shallow drilled wells in Ohaba-Forgaci (0.082 ng/ml) and Dudeștii Noi (0.075 ng/ml) were included in the category of drinking water, although these sources serve to water animals and for the irrigation of garden crops. Out of these two, only the water sample from Ohaba-Forgaci was quantifiable. Studies concluded that antibiotic residues from soil or water can be taken up by vegetables through a passive absorption mechanism and water transport. The highest concentrations of antibiotics in vegetable tissues were found in the leaves, then in the stems, and the lowest was found in the roots (Hu X. *et al*, 2010).

Balzer et al. (2016) recommended a threshold value of 0.1 ng/ml for antibiotic residues in groundwater. Exceeding this value would mean that the substance is in high concentrations and presents a risk factor due to persistence, bioaccumulation, or toxicity (Balzer F. *et al*, 2016).

In the case of Ohaba-Forgaci and Dudeștii Noi, it can be observed that the values found in water from the shallow drilled wells (maximum 10 meters) are identical to those obtained from the public distribution network of the same localities, which, according to the local authorities, were drilled deeper (minimum 100 meters). As there were no differences between the water from shallow-drilled wells (10 m) and deep-drilled wells (100 m), we can assume that this is due to human activity, more specifically the quality of the drilling.

The amount of residue decreases in the Timiș River from upstream to downstream during the period with increased water flow (S II). The values go from 0.105 ng/ml downstream Caransebeș to 0.089 ng/ml at the entrance of the Lugoj drinking water treatment plant. The same trend is observed in the first period of sampling (S I). Even when the residue values were below the limit of quantification, they decreased from 0.079 ng/ml upstream Lugoj water treatment plant to 0.078 ng/ml in tap water from this plant. In the Bistra River, higher residue concentrations (0.115 ng/ml) were detected than in the Timiș River. Water sampled from private wells in Ciuta, located to the right of the Bistra River, had higher concentrations than the river water, namely 0.132 and 0.138 ng/ml.

In the Bega River, the residue concentration decreased during the period with heavy rainfall (S II) from 0.099 ng/ml in the sampling point from Făget to 0.097 ng/ml in Bucovăț. Downstream, in the samples collected from the public drinking water network of Timișoara (potable water resulting after treatment of surface water from Bega River), we found very high concentrations (0.276 ng/ml), compared to all other drinking water sources. The beta-lactam residue concentrations from the water of the Bega River at the entrance to the water treatment plant (0.093 ng/ml) as well as at the collection point in the city center of Timișoara (0.087 ng/ml) were quantifiable even in the first stage of sampling, during normal weather (S I).

Although there are studies that claim that certain drinking water treatment technologies, such as ozonation, the use of chlorine dioxide, and UV irradiation, can reduce the load of pharmaceutical residues, very few of them are considered relevant, hence the results have to be interpreted with caution. Improvements in the experimental conditions are needed for the accuracy of the recreation of the phenomena that take place at each stage of treatment. Furthermore, the concentrations at which certain substances specific to the treatment method are used, such as oxidizing agents, must be taken into account for the recreation of different technologies. What is known with certainty is that the oxidation of some pharmaceutical substances can result in compounds that are more toxic than the original structures (Charuaud L. *et al*, 2019). The high concentration of beta-lactam residues found in the public distribution network in Timișoara can be ascribed to the lack of surveillance in the area upstream of the city's water treatment plant.

The river Mureș, in contrast, showed an increase in the residue concentration from upstream to downstream. The values change from 0.075 ng/ml (below the limit of quantification) in Săvârșin to 0.098 ng/ml in Lipova. Intermediate values of 0.09 and 0.087 ng/ml were detected in samples from private drinking water wells in Țela, located on the left side of the Mureș River, between Săvârșin and Lipova. At the time of sampling (S II), the water level in these wells was increased as well.

During the second sampling period, characterized by heavy rainfall and flooding, higher values for beta-lactam residues were recorded, regardless of the water category. The increased values can be attributed to floods that have collected residues of any kind from the respective areas, counteracting the dilution effect that the increased amount of water would have normally demonstrated. The presence of residues in floods can be attributed to the education, or lack thereof, of the inhabitants in regard to the environment or their understanding of their responsibility towards it.

Although a considerable number of valuable studies on residues of pharmaceuticals in aquatic environments, among which antibiotics hold an important position (Lamastra L. *et al*, 2016; Meffe, R. *et al*, 2014; Szekeres E. *et al*, 2018), have emerged in Europe over the past 10 years, the watch list developed by the European Commission for the Union-wide monitoring of hazardous substances currently includes only five antimicrobials, namely erythromycin, clarithromycin, and azithromycin of the macrolide class, amoxicillin from the beta-lactam class and ciprofloxacin from the fluoroquinolone class (EU Commission Implementing Decision, 2018).

CONCLUSIONS

Residues of beta-lactam antibiotics were identified in all categories of water (surface water, wastewater, and groundwater) in the territory of Timiș County.

The largest amount of residues, excluding wastewater, was detected in the tap water sample from the public distribution network in the city of Timișoara. This can be attributed to the lack of surveillance for emerging organic contaminants upstream of the city's drinking water treatment plant.

During the period of heavy rainfall, due to the formation of floods, the amount of residues increased in all categories of water.

The antibiotic residue removal efficiency of the wastewater treatment plants in this study was 75.18% for Timișoara, 68.57% for Jimbolia, and 23.61% for Dudeștii Noi.

Enzyme-linked immunosorbent assay can be successfully used as a screening tool for the presence of antibiotic residues in aquatic environments.

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SYNCHRONIZATION OF OVULATION (FTET) IN TURCANA SHEEP AS EMBRYO RECIPIENTS

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Abstract

Due to the fact that the farm wants to crossbreed sheep with meat breeds, it was opted for the fastest solution to bring genetic progress, namely embryo transfer, using sheep from the Turcan breed as embryo recipients and those from the Suffolk breed as embryo donors, so that finally the batch of receivers after parturition will produce a production of Suffolk lambs. Following the selection of recipients considering the standard criteria that include: general health, functional integrity of the genital tract and cyclic activity of the ovaries, two groups were formed for the induction and synchronization of estrus. The first batch consisted of 20 sheep and the second batch of 20. The first batch was subjected to a P4-PG-PMSG protocol and the second batch to the P4-PG-GnRH protocol. The two protocols consisted of the insertion of intravaginal sponges with flugestone acetate in a concentration of 20mg, to induce the progesterone phase, for 13 days, day 0 of the protocols being represented by the day when the progesterone sponges were applied. In continuation of the protocol, Prostaglandinf2α was used on day 12 in a double dose, in the morning and in the evening at a distance of 12 hours in a dose of 0.6 ml/administration/animal. On day 13, the progesterone sponges were removed and PMSG (Folligon) was administered in the case of the first batch at a dose of 600 IU/animal, and in the second batch GnRH (Receptal) was administered at a dose of 12 μg/animal (3ml/animal) followed by a dose of 600 IU HCG (Chorulon) 24 hours after GnRH administration. Ovulation was accurately assessed at the time of embryo transfer through laparotomy, due to the highlighting of the ovaries and their macroscopic analysis in the operative field, after identifying the CL on the ovary, their appearance and the number of CL on the ovary. However, the assessment of the rate of entry into heat was assessed 24 hours after the end of the protocol by biostimulating the receptors with the help of detector rams, thus the ewes that entered in estrus at 12 – 24 hours, 24 – 48 of hours or over 48 hours after completing the protocol. The results obtained in the case of the first batch having the highest rate of entering in estrus between 24 - 48 hours being 40%, followed by a rate of 30% between 12 - 24 hours, the lowest rate being 20% that entered in estrus after 48 hours, the second batch with GnRH had the highest rate of entry into estrus of 60% in the first 12-24 hours and the rate at 24-48 hours, respectively those that entered heat after 48 hours was 20%. Compared between the two groups, there were differences in the timing of ovulation assessment, as in the case of the first group with PMSG, a 60% ovulation rate was assessed with well-developed CL, 10% presented CL but these were unsuitable for embryo transfer, 10% presented ovarian cysts, and 10% did not ovulate, in the case of the second batch an ovulation rate of 60% was assessed and the rate of 40% represented the animals that did not ovulate, the difference between the two batches being the fact that it is observed in the case of the first batch rate of 10% with ovarian cysts, which indicates that the PMSG-based pharmaceutical is causing ovarian cysts. In conclusion, the therapeutic protocol used in the off-season for the induction of estrus and ovulation in Turcan sheep, in this study, resulted in the detection of estrus in 90% of the ewes subjected to the protocol, and the ovulation rate was 80% (60% with CL well developed and 20% were with poorly developed CL), regarding the first batch, and regarding the second batch 100% of ewes in oestrus were detected, but 60% of them ovulated with CL well developed. These results can be largely attributed to the reproduction seasonality of the sheep.

Key words: Țurcana, Embryotransfer, Receptors, Synchronization, Ovulation.

1. INTRODUCTION

The aim of the study was to induce and synchronize oestrus in Turcan sheep, in order to transfer embryos from the batch of donor sheep, from the Suffolk breed, to bring rapid genetic progress on the farm, in the off-season of sheep reproduction.

Regarding the success of assisted reproduction in the off-season, the farm also intervened with natural stimulation to imitate as much as possible the natural breeding season. In this sense, the light-dark ratio and the optimal temperature for reproduction in sheep were adapted.

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The objectives of this study were the following:

• synchronizing the hormonal status of recipient sheep with the objective of survival, development and hatching of embryos from donor sheep.

• it is aimed that the recipient female is synchronized with the age of the embryo;

• calculation of ovulatory timing between donors and receivers, group synchronization of Țurcana receivers in the off-season.

• real estimation of ovulation through laparotomy and embryo inovulation (ET) 6 - 6.5 days after the onset of heat.

2. MATERIALS AND METHOD

2.1. Animals taken in the study

This study was carried out between June 25 and July 16, 2021, during the natural breeding off-season. The farm having a flock of 300 Turcana sheep, 2 batches of sheep were selected, one batch of 20 ewes and the second of 20, for the induction and synchronization of estrus according to the reproductive status (number of calvings, number of calvings with dystocia, degree of development of the female genital system, genital system conditions), as well as by maintenance status, body score, health status (comorbidities, possible infectious diseases, parasitic diseases, foot conditions). The selected ewes are lambs and primiparous, having BCS 3-4, with a weight of approximately 40-45 kg, and from a clinical point of view being healthy.

The sheep were kept in the stable, being fed in the specially arranged box, the fodder ration being made up of coarse, juicy and concentrated in a balanced ratio, in order to stimulate the reproduction and the needs of the animals, water being administered ad libitum. The light in the shelter was artificial in order to adjust the light-dark ratio, thus the selected animals were exposed to 12 hours of light and 12 hours of darkness (ratio 1:1), according to the natural winter season (autumn).

2.2. Off-Season Estrus Induction and Timing Protocol

In the natural off-season of breeding (spring and summer), as well as in animals that do not show signs of estrus for 20 days or more from the beginning of the breeding season or remain non-pregnant after the first breeding/insemination, it is necessary to stimulate sexual behavior and to initiate gestation as soon as possible (Pavlo Sklyarov et al., 2021).

Inducing oestrus during the summer or spring provides the additional chance to treat young ewes that have aborted or had non-viable lambs. Another goal is to shorten calving intervals to achieve three lambs in 2 years or 2 lambs per year (Pavlo Sklyarov et al., 2021).

To induce estrus in small ruminants during the off-breeding season, administration of progestin is the method of choice and causes prolongation of the luteal phase in these animals. As a result, they are synchronized and, after the cessation of hormone treatment, the animals enter estrus simultaneously. (Ajbazov et al., 2006; Aksenova et al., 2012).

The sheep selected for the study were grouped into two groups, the first group consisting of 20 sheep and the second group of 20 sheep. The two batches were subjected to two different estrus induction and timing protocols.

The first batch taken in the study were administered on day 0 intravaginal sponges with flugestone acetate (cronolon) in a dose of 20 mg, which have a progesterone effect, for 13 days. One day before the extraction of the sponges, they were treated with 2 doses of ProstaglandinF2α, administered in the morning and in the evening 12 hours apart, each 0.6 ml/dose/animal. On day 13 of the protocol, progesterone sponges were extracted and PMSG (Foligon) was administered at a dose of 600 IU/animal. 48 hours after the extraction of the sponges, the ewes were subjected to a biostimulation with the help of experimental rams, which were previously prepared not to intrude by applying an apron covering the furrow, identifying the ewes in estrus and dividing them according to how long has it been since the sponge extraction. The ewes taken in the study were not mounted or artificially inseminated in order to submit them to embryo transfer after 6 - 6.5 days after ovulation.

For the second batch, the intravaginal sponges with flugestone acetate (cronolon) in a dose of 20 mg were maintained as in the first batch for 13 days. On the 12th day of the protocol, ProstaglandinF2α was administered at 12-hour intervals in a dose of 0.6 ml/dose/animal. On day 13, the intravaginal sponges were removed and instead of PMSG as in the case of the first batch, the sheep received 12 μg/animal (3 ml/animal) of GnRH (Receptal), and 48 hours after the extraction of the sponges administered 600 IU of HCG (Chorulon), then the same procedure was followed as in the case of the first batch.

3. RESULTS AND DISCUSSIONS

3.1. Estrus expression and diagnosis in embryo-receiving Turkish sheep

Due to the fact that one of the main objectives of the study was the induction and synchronization of sheep from the Turcan breed, in the off-season, in order to use them in the embryo transfer protocol as recipients of embryos from Suffolk breed donors, ovulation was accurately assessed at the moment embryo transfer through laparotomy, due to the highlighting of the ovaries and their macroscopic analysis in the operating field, after identifying the CL on the ovary, their appearance and the number of CL on the ovary. But the assessment of the rate of entry into heat was assessed 24 hours after the completion of the protocol by biostimulation of the receptors with the

help of experimental rams, the ewes that entered estrus at 12 - 24 hours, 24 - 48 hours or more were assessed 48 hours after completing the protocol (Table 1).

Table 1

Appreciation of ewes in heat, from batch 1, in different periods of time

12-24h	24-48 h	> 48h
30% (6/20)	**40% (8/20)**	**20% (4/20)**

According to Table 1 a percentage of 90% (18/20) of the ewes in the first batch selected for the estrus induction and synchronization protocol went into heat, 10% (2/20) did not go into estrus. Their distribution according to the time period since the completion of the induction and synchronization protocol and the percentage in each time category can be found in Figure 1.

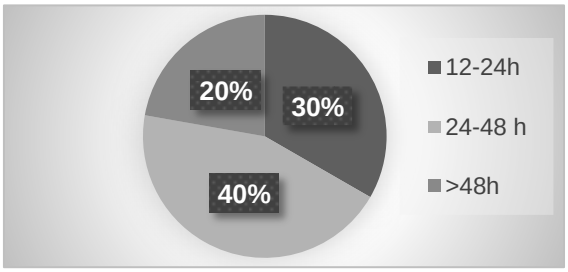

Figure 1 - **The percentage of ewes diagnosed in oestrus, from batch 1, in different time periods**

From the 20 ewes receiving embryos, from the Țurcana breed, selected in the first batch, following their identification according to the time period in which they entered estrus, a percentage of 30% were identified in the first 12-24 hours from finalization of the estrus induction and synchronization protocol, respectively 40% between 24-48 hours and 20% after 48 hours.

Table 2

Appreciation of ewes in heat, from batch 2, in different periods of time

12-24h	24-48 h	> 48h
60% (12/20)	**20% (4/20)**	**20% (4/20)**

The ratio of ewes in group 2, which were synchronized with GnRH, according to the time period can be seen in Figure 2. related to Table 2., so that 60% (12/20) entered heat between 12 – 24 hours, 20% (4/20) between 24 – 48 h, respectively 20% (4/20) after 48 hours.

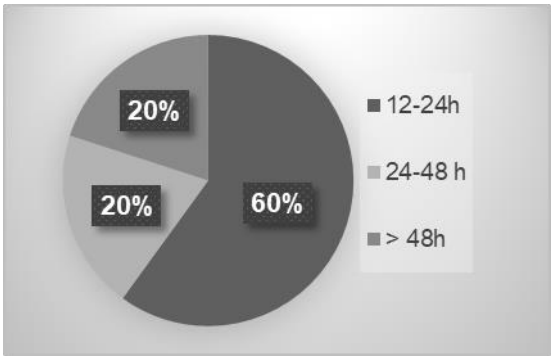

Figure 2 - **The percentage of ewes diagnosed in oestrus, from batch 2, in different time periods**

Comparing the two treatment regimens for estrus stimulation, we believe that both produced the expected results according to the specialized literature. Zamfirescu Stela at the Sheep and Goat Research Institute in Palas, Constanța obtained an estrus synchronization rate between receivers and donors between 80-95%, but in mestizo sheep between Tsiigai and Merino. The results obtained through this study show that these synchronization methods are validated and can be successfully used in other sheep breeds, the total mean of estrus induction 0% Turcana sheep was 95%, with limits between the two batches 80% at lot 1 and 100% for lot 2.

The difference between the groups is 20% in favor of group 2, it seems that the administration of GnRH on day 13 and hCG on day 15 led to the efficiency of estrus induction in Turcane.

Also in batch 2, the best grouping of estrus in the 12-24 hour interval was obtained, this being 60%. In ET sessions, a gap of maximum 12 hours is accepted between the estrus expressed between donors and recipients.

3.2. The actual ovulation rate of ewes of the Turcana breed

Following the embryo transfer protocol, the method used in small ruminants being the surgical one, through laparotomy, the presence and macroscopic appearance of CL on each ovary were highlighted, thus only the females that presented CL, on day 6 - 6.5 after ovulation, well developed have benefited from embryo transfer.

Following the surgery, it was found in the first batch of sheep used as embryo recipients (Figure 3) that 60% presented a well-developed CL, representing the fact that they had ovulated, but 20% ovulated but the CL was poorly developed following follicular dehiscence making these females unsuitable for embryo transfer, 10% of the possible recipients did not respond to the treatment

as CL was not identified on the ovaries, and in 10% of cases the presence of follicular cysts on the ovaries was identified (2/20).

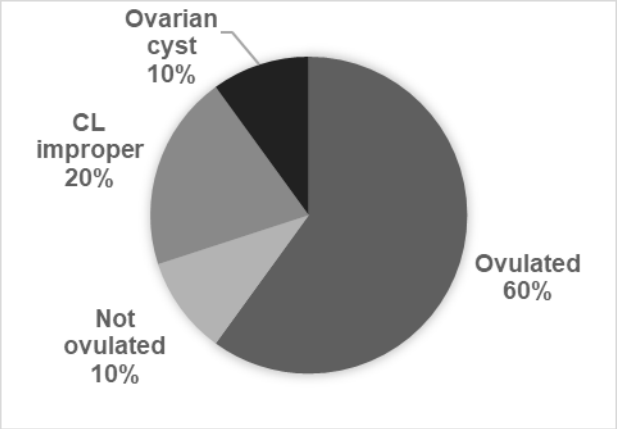

Figure 3 - **Ovulation rate of embryo recipients, from the first batch, following laparotomy**

In the second batch of embryo recipients, in which GnRH was used for estrus induction and synchronization, an ovulation percentage of 60% was found (Figure 4) with well-developed CLs on the ovaries, the respective females being used for embryo transfer and 40% of these did not show CL on the ovary which demonstrated that those females did not ovulate.

We conclude that the actual ovulation rate required for successful ET, assessed laparoscopically, was 60% and was identical in both groups. We note that in batch 2 no ovarian cysts were identified and no poorly developed CLs, the ovulations produced generated very qualitative CL. Due to the percentage of 40% of ewes that did not ovulate, although they were in oestrus (batch 2), it is necessary to increase the dose of hCG administered on the 15th day and change it with GnRH to force the ovulation of more follicles develop.

In the second batch of embryo recipients, in which GnRH was used for estrus induction and synchronization, an ovulation percentage of 60% was found (Figure 4) with well-developed CL on the ovaries, the respective females being used for embryo transfer and 40% of them did not present CL on the ovary, which demonstrated that those females did not ovulate.

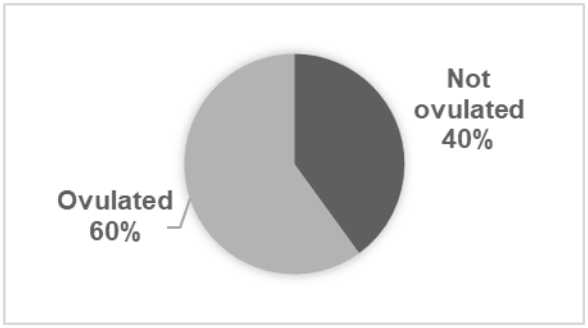

Figure 4 - **Ovulation rate of embryo recipients, from the second batch, following laparotomy.**

3.3. Discussions

Biehl et al. showed that estrous behavior in sheep appeared mainly within 48 h after withdrawal of the progestogen sponge (Xiaojie Yu et al., 2022), which is also found in this study. The estrus induction rate in the case of sheep from the Țurcana breed is the highest in the case of the first batch between 24 - 48 hours after the extraction of the sponges, followed by the interval of 12 - 24 hours. In the case of the second batch, most of the ewes entered estrus 12-24 hours after the extraction of the sponges.

Xiaojie Yu et al. showed that PMSG has direct action on the ovary, but it often caused ovarian cyst, even though it effectively facilitates follicular development in terms of induction and timing of ewes, thus affecting AI and embryo transfer (Xiaojie Yu et al. ., 2022). This is also found in this study in the ewes synchronized with PMSG from the first batch, having a share of 10% of the ewes with ovarian cysts, respectively 20% of the ewes synchronized with PMSG although they ovulated and developed CL on the ovary they have were inappropriate for the embryo transfer protocol. Xiaojie Yu et al., 2022 demonstrated that GnRH and its analogues through its action on the pituitary gland generate the secretion of FSH and LH without a difference in the rate of induction and synchronization compared to PMSG-synchronized ewes, but in in the case of ewes synchronized with GnRH, a shortening of the elapsed time from estrus to ovulation was observed, which was also observed in this study, in the second batch 60% of the ewes synchronized with GnRH ovulated and were used in the embryo transfer protocol , but estrus was mostly manifested 12-24 hours after progestogen sponge extraction in a percentage of 60%, no ewes with ovarian cysts were identified in this study following their synchronization with synthetic analogue of GnRH.

4. CONCLUSIONS

The protocol for inducing estrus and synchronizing ovulation in embryo recipient sheep aims at synchronizing their hormonal status with the objective of survival, development and hatching of embryos from donor sheep. The aim is for the recipient female to be synchronized with the age of the embryo, practically a perfect overlap of the time elapsed since ovulation and the degree of development of the transferred embryo is desired. At the end of this protocol, after 6-6.5 days from estrus, instead of AI, the embryo collected from the donor female will be directly inovulated (ET). Considering the fact that the sheep is a seasonal polyestrous animal (estrus occurs in autumn and spring), with the help of this protocol we make it possible to induce estrus and induced ovulation, and thus it is possible to carry out the transfer of embryos outside the breeding season. 10 Turcana sheep were selected as possible embryo recipients. Estrus induction in the off-season was carried out through a hormonal protocol: P4-PG-PMSG. Progesterone (P4) was administered for 13 days in the form of vaginal inserts (Chronogest), on the 12th day a dose of PG was administered, and on the 13th day of treatment PMSG (Folligon) was administered in dose of 300 IU. The ovarian response to follicular stimulation and the induction of the estrous phase was assessed starting 24 hours after the end of the therapeutic scheme, by using detection rams. Thus, 90% (9/10) of the receptors were diagnosed in estrus with their distribution as follows: 30% (3/10) between 12-24h, 40% between 24-48 hours and 20% (2/10) after 48h of to treatment. At the time of using the receptors in the ET protocol (6.5 days after estrus), ovulation was accurately assessed, according to the appearance and identification of the CL on the ovary. Of the total number of recipients in the ET protocol, 60% ovulated, 30% did not respond to treatment (without CL, did not ovulate) and in 10% of cases ovarian cysts were identified (1/10). The therapeutic protocol used to induce estrus and ovulation in Turcan sheep, in the off-breeding season, had an effect of 90% estrus and 60% ovulation rate.

The therapeutic protocol used in the off-season to induce estrus and ovulation in Turcana sheep, in this study, resulted in the detection of estrus in 90% of the ewes subjected to the protocol, and the ovulation rate was 80% (60% with well-developed CL , and 20% were with poorly developed CL), regarding the first batch, and regarding the second batch, 100% of ewes in estrus were detected, but 60% of them ovulated with well-developed CL. These results can largely be attributed to the seasonality of sheep reproduction.

The difference between the groups is 20% in favor of group 2, it seems that the administration of GnRH on day 13 and hCG on day 15 led to the efficiency of estrus induction in Turcane.

We conclude that the actual ovulation rate required for successful ET, assessed laparoscopically, was 60% and was identical in both groups. We note that in batch 2 no ovarian cysts were identified and no poorly developed CL, the ovulations produced generated very qualitative CLs. Due to the percentage of 40% of ewes that did not ovulate, although they were in oestrus (batch 2), it is necessary to increase the dose of hCG administered on day 15 and change it with GnRH to force the ovulation of more developed follicles.

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EFFECTS OF ANTIOXIDANT TREATMENT ON CELL DIFFERENTIATION IN RABBIT EMBRYOS

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Abstract

The antioxidant coenzyme Q10 can influence the expression of genes involved in apoptosis and energy metabolism of oocytes and quercetin can improve oocyte maturation and early embryonic development. In this study, the gene expression of *GATA6* and *NANOG* in rabbit embryos was assessed using the qRT-PCR reaction. The groups were: group A- control group (no treatment added), group B (hormonal treatment of superovulation, which included the administration of PMSG and hCG), group C (administration of quercitin) and group D (administration of Coenzyme Q10). Our results show that the expression of the two genes was different depending on both the stage of embryonic development and the treatment administered. The highest values of gene expression for GATA6 and NANOG were obtained in groups 2, 4, 7, 8 and 9, corresponding to morula and blastocyst stages. In addition to the fact that NANOG and GATA6 are factors that are involved in early embryonic development, we believe that the administration of extrapituitary gonadotropins and antioxidants contributed to the increase in gene expression.

Key words: rabbit embryos, gene expression, antioxidants, qR-PCR

The female rabbit has certain anatomical and physiological characteristics relevant to embryology research and the application of assisted embryo reproduction techniques.

Ovulation in the rabbit is induced by mating. Coitus leads to the nervous stimulation of the vagina, thus triggering the production of gonadotropin releasing hormone (GnRH) in the hypothalamus. Thus, under the influence of this hormone, the anterior pituitary gland secretes the follicle-stimulating hormone (FSH) and the LH (luteinizing) hormone responsible for inducing ovulation.

Q10 is an endogenous antioxidant that protects against oxidative damage and regulates gene transcription. Coenzyme Q10 supplementation may also have beneficial effects on ovarian reserve and follicular development. It also has the ability to restore mitochondrial function by reducing mitochondrial membrane damage and depletion of the intracellular antioxidant ATP. Q10 can influence the expression of genes involved in apoptosis and energy metabolism of oocytes. By reducing the expression of pro-apoptotic genes and increasing the expression of anti-apoptotic genes, Q10 protects oocytes from apoptosis and contributes to maintaining their integrity.

Quercetin can improve oocyte maturation and early embryonic development. This substance was also found to have the ability to reduce apoptosis and enhance autophagy in aged oocytes, providing a protective mechanism against age-related mitochondrial oxidative stress.

The GATA family represents a family of transcription factors of fundamental importance in gene regulation and organismal development. The *GATA6* gene is expressed since embryogenesis, even from the early blastocyst stage at 3.5 days. GATA factors, including GATA6, regulate the expression of cardiac and smooth muscle genes, having an essential role in the processes of development, differentiation and gene expression. They interact directly with GATA(A/G) DNA sequences in the promoters and enhancers of target genes, thus regulating their expression.

NANOG is an essential transcription factor in early embryonic development and in the maintenance of stem cell pluripotency. Its functions are crucial in the specification of pluripotent epiblast (EPI) cells at the expense of primitive endoderm (PrE) within the inner inner cell (ICM) of the blastocyst.

Interactions between NANOG and GATA6 are essential in controlling cell specification within the ICM. At the 16-cell stage, all ICM cells express both *NANOG* and *GATA6*, forming a population of double-positive (DP) cells. These close interactions between *NANOG* and *GATA6* are crucial in regulating the process of cell

specification and cell plasticity in the early embryo.

In this study, we evaluated the expression of *GATA6* and *NANOG* genes in rabbit embryos administered antioxidants, such as coenzyme Q10 and quercitin.

In this context, the aim of this paper was to determine if the treatment of females with hormones or antioxidants influences the expression of genes involved in cell differentiation processes in early embryos.

MATERIAL AND METHOD

For this study, we used New Zealand female rabbits. The selected females were 4 months old and had a body weight between 3 and 3.3 kg at the time of the experiment. To ensure a suitable environment, all females were reared in individual cubicles, where they benefited from natural light and were exposed to an ambient temperature between 15 and 25°C. During the experiment, the rabbits had unlimited access to water and were fed with commercial food in the form of pellets.

Table 1.

The embryonic stages of the groups analyzed

Experimental group	Embryonic stage	Stimulated/Non-Stimulated
A1-A3	7 morulae 12 blastocysts	Stimulated with male
A5-A8	19 morulae 7 blastocysts	Non stimulated
B1-B4	9 blastocysts	Stimulated with male + hormones
B1-B4	24 morulae	Stimulated with male + hormones
B1-B4	27 morulae degraded	
B5-B8	19 blastocysts	Stimulated with hormones
C1-C8	43 morulae	Stimulated with Q10
C1-C8	14 blastocysts	
D1-D8	17 morulae 2 blastocysts	Stimulated with querctin

In this study, the rabbits were grouped into four groups, each group having 8 females. The description of the experimental groups is as follows: no treatment added (group A- control group), hormonal treatment of superovulation, which included the administration of PMSG and hCG (group B), administration of quercitin (group C) and administration of Coenzyme Q10 (group D).

Group B received 120 IU/female of a PMSG analogue (Folligan, MSD) intramuscularly, then 180 IU/female of HCG (Chorulon, MSD) at 48 hours, but 6 hours before mating.

In group C, quercetin was administered orally for 20 days, at a dose of 30 mg/kg (using Quercetin 500mg/Solaray).

Group D received oral CoQ10 for 20 days at a dose of 10 mg/kg. For groups C and D, oral administration was performed daily at the same time interval using a syringe.

Embryo harvesting was performed by washing the oviducts and uterine horns with a PBS solution (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate buffer / 100 mL, VWR Life Science), after performing the ovariohysterectomy.

Gene expression analyses

Protein sequences were transcribed into mRNA sequences. These mRNA sequences were subsequently transcribed into cDNA sequences. To identify the indicator genes of the cell differentiation process, the sequences of the specific primers were taken from the specialized literature. The oligonucleotides of these primers were synthesized in the Eurogentec laboratories in Belgium and screened for use in this study. The primers sequences used in this study were: *GATA6* Forward 5' TGCGGCATCTACAGCAAGAT 3' and Reverse 5' CCCGGCCCATTGTTTCCT 3'.

For the extraction and purification of total RNA from the samples, the SV Total RNA Isolation System kit (Promega, USA) was used.

To verify the quantity and quality of the extracted RNA, the spectrophotometric method was used using the Nanodrop 8000 UV-VIS spectrophotometer (Thermo Scientific). Based on the results obtained for RNA, the amount required for the reverse transcription reaction was calculated.

For cDNA synthesis, the Hight-capacity cDNA Reverse Transcription kit (Thermo Scientific, Lithuania) was used. The required solution was prepared and transferred to ice. 10 µl of RNA was added to the prepared solution and mixing was done gently. The mixture was then transferred to a thermocycler.

The synthesis and amplification program consisted of keeping the samples for 10 minutes at 25°C, then at 37°C for 120 minutes. Reverse transcriptase activity was stopped by keeping the samples for 5 minutes at 85°C.

For qPCR reactions, the GoTaq qPCR Master Mix Kit (Promega, USA) was used. This is a ready-made solution optimized for the quantitative PCR amplification reaction. The kit contains a DNA polymerase enzyme (GoTaq DNA Polymerase) and dNTPs (nucleotide triphosphate) in a buffer optimized for PCR. It also contains a dye, SYBR GREEN, and is supplemented with a passive reference dye called ROX.

Each sample was analyzed in two replicates. To have a control for each individual primer, a sample without cDNA template was analyzed.

The gene expression ratio was normalized using the expression value of the β-actin gene, which is a constitutively expressed gene. For each sample, the number of threshold cycles (Ct) was determined. The method used for relative quantification was the Δ (ΔCt) method. According to this method, the relative ratio (R) between the control and the stressed variant was calculated using the following formula: $R = 2^{(-\Delta\Delta Ct)}$.

The obtained results were statistically interpreted using the Microsoft Excel program and ANOVA (Analysis of Variance).

RESULTS AND DISCUSSIONS

GATA6 gene expression values and their interpretation

GATA6 is an essential gene in early embryogenesis, especially during the blastocyst period, being expressed in mesoderm and endoderm derived tissues (3,4).

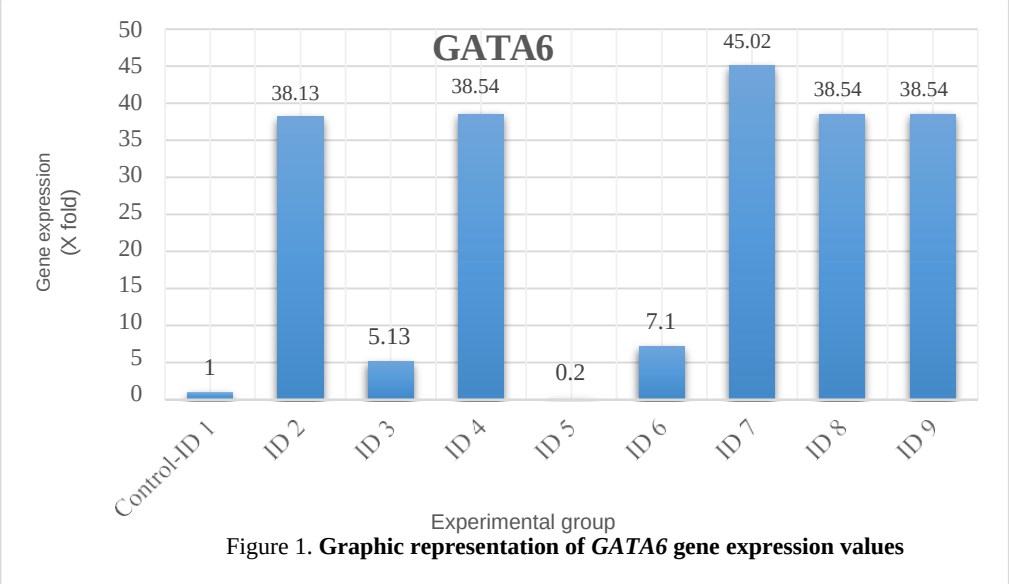

Figure 1. **Graphic representation of *GATA6* gene expression values**

In our study, the expression of this gene was identified in all 9 analyzed samples. As can be seen from Figure 1 there are differences between the expression levels. Thus, considering group 1 as a control, embryos obtained from females after stimulation with the male, which was given the value 1 and in comparison, with this unstimulated group 2 shows a gene overexpression, due to the fact that embryos were found in this group in morula and early blastocyst stage. This could be explained by the fact that GATA6 is the earliest PrE (primitive endoderm) gene to be expressed. Also, *GATA6* gene is present in all blastomeres from the eight-cell stage, alongside *NANOG*, *OCT4* and *CDX2*. (Dietrich et al., 2007)

In the case of group 3, the gene expression is higher compared to the control group, but lower than in the case of group 2, because this group consists only of blastocysts in which to measure the advancement of the embryonic stage, the expression value of this gene is lost.

Regarding group 4, the embryonic stage is late morula, when the cell differentiation process is initiated, which explains the overexpression of this gene.

Group 5 was composed of degraded morulae in which the processes of embryonic development are seriously slowed down, so that the expression of genes stimulating cellular differentiation is no longer possible, studies supporting the fact that embryos lacking gene expression die during gastrulation (Zhao et al., 2005), fact that explains such a low expression of the *GATA6* gene.

***NANOG* gene expression values and their interpretation**

The *NANOG* gene is an important gene in embryonic development and in the maintenance of stem cell pluripotency. *NANOG* is part of the family of transcription factors and is expressed in embryonic stem cells as well as other pluripotent cell types. This gene plays an essential role in maintaining pluripotent cell identity and controlling cell differentiation (Khang et al., 2017).

The data in figure 2 presented below show that in group 2 that was not stimulated, an overexpression of the *NANOG* gene can be observed due to the fact that the embryonic stage analyzed is in the optimal period of expression, more precisely in the early blastocyst stage.

Decreased *NANOG* gene expression in the early blastocyst results in embryonic death. However, Chambers et al. (2007) support the fact that embryonic stem cells in which the *NANOG* gene is weakly expressed are still capable of maintaining pluripotency, although they are prone to differentiation.

At the same time, comparing the graph data, we can see that there is an overexpression of

the gene also in group 4 (stimulated with male and hormones).

The development and maturation of ovarian follicles is dependent upon the successive actions of gonadotropines. Upon stimulation of immature antral follicles by FSH, there is an upregulation of expression levels of both aromatase and LH receptor mRNA. Subsequently, LH acts directly or indirectly through the actions of growth factors (Park et al., 2004) on the FSH stimulated follicle to facilitate steroid production, induce luteinisation and ovulation.

Activation of LH receptors also contributes to the upregulation of oviductal glycoprotein (OGP). The activation of LH receptors in the oviduct causes an increase in the synthesis of OGP that binds to the embryo and helps its growth and development. (Zheng et al., 2001)

An overexpression of the gene is also evident in groups 7 and 8 (stimulated with coenzyme Q10), but also in the case of group 9 (stimulated with quercitin), a fact that supports the theory that *NANOG* expression occurs in the early stages of the morula (Kang et.al, 2017).

NANOG is expressed in a limited number of cell types and only in cells that also express OCT4, including ESCs. NANOG is located in the center of the morula and in the ICM (inner cell mass) of the blastocyst.

An overexpression of the gene is also evident in groups 7 and 8 (stimulated with coenzyme Q10), but also in the case of group 9 (stimulated with quercitin), a fact that supports the theory that NANOG expression occurs in the early stages of the morula.

Also, the antioxidants protects the ovarian reserve, counteracts the physiological aging of the ovaries by restoring mitochondrial function and increases the rate of embryo cleavage and blastocyst formation.

In groups 3 and 6, according to the statistical analysis graphically represented in figure 2, the expression of the *NANOG* gene is weak, which reinforces the current knowledge that with the advancement of the embryonic stage the expression is suppressed in the early blastocysts, with the induction of ICM differentiation into EPI. The lowest value of NANOG expression was recorded in group 5, in which there are degraded morulae.

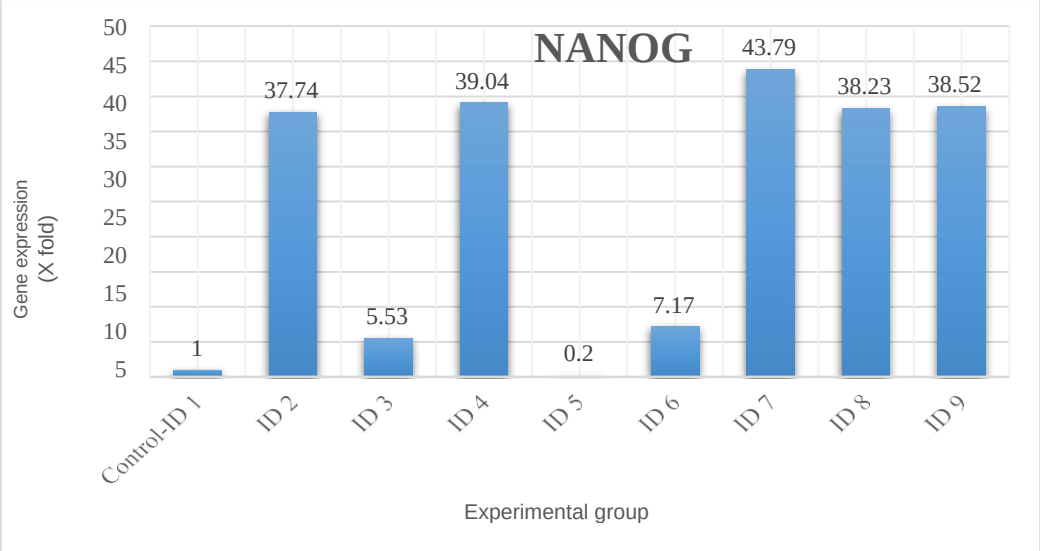

Figure 2. **Graphic representation of *GATA6* gene expression values**

CONCLUSIONS

The overexpression of GATA6 and NANOG genes was recorded in cases of groups 2, 4, 7, 8 and 9, which were in the morula and blastocyst stages. In the case of the groups to which the analogue hormones of FSH and LH and antioxidants were administered, an overexpression of the *GATA6* and *NANOG* genes was found, with the exception of group 5 in which there was a degraded morula stage.

The obtained results showed that hormonal and antioxidant treatments can influence the expression of certain genes involved in embryonic development, but these aspects are also closely correlated with the stage of development in which the embryo was captured.

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