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CONTENTS

THE ROLE OF VIRAL INFECTIONS IN THE DEVOLOPMENT OF RESPIRATORY DISEASE IN SWINE

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Abstract
Susceptible categories of pigs for the occurrence of respiratory diseases are categories in postweaning and fattening. The spectrum of clinical symptoms is very wide and includes not only respiratory symptoms, but also many other symptoms that appear as a consequence of systemic disorders, depending on the type of causative agent. Cough can be of different frequency, intensity and productivity, and difficulty breathing can be of different degrees. Respiratory diseases may be accompanied by stunted growth, rough bristles, anorexia, reduced daily gain and lethargy. Disseminated systemic disorders often include symptoms suggestive of central nervous system disorders, swollen joints and lameness, followed by reproductive disorders or sudden death. Pathomorphological changes are also of different character and severity. Non-specific factors predispose to the occurrence of initial damage or contribute to the further spread of already existing lesions. In this article, we wanted to give an overview of our research on respiratory diseases of viral etiology in pigs from commercial farms.

Key words: pigs, respiratory diseases, viral infections

The primary causative agents of swine respiratory diseases are PRRSV, swine influenza virus (SIV), porcine circovirus type 2 (PCV-2), *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae, Bordetella bronchiseptica*, and in some cases the virus that causes Aujeski's disease (Pseudorabies Virus - PRV) and the respiratory corona virus (Porcine Respiratory Corona virus - PRCV). Recently, it has been reported that among the infectious agents, the porcine reproductive and respiratory syndrome virus and *M. hyopneumoniae* play a particularly significant role in the etiopathogenesis of respiratory infections (Ivetić V.et.al. 2000, 2005, Došen R.et.al. 2007, Savić B. et.al. 2010, 2012 a,b).

It is characteristic that many of the respiratory pathogens can appear as independent causative agents, or, which is more common, mutually associated in a synergistic action. Their spread and frequency of occurrence varies, both from region to region, and within the region itself, depending on the technological-production group. Many of the mentioned pathogens can be found simultaneously in the same pig farms. In addition to the above, this is also the reason that their classic division into primary and secondary respiratory pathogens is not always completely acceptable (Šamanc, 2009). If it is known that some causative agents, previously designated as secondary pathogens, such as *Pasteurella multocida*, can independently cause the disease and are then considered a primary pathogen (Lončarević, et.al. 1997, Šamanc H. 2009). The interaction of pathogens is very complex, and each of them independently, in synergism or competition, causes a certain manifestation of the disease from the respiratory complex (Radojičić S. et.al. 2011).

Special importance is also attached to non-infectious, i.e. predisposing factors, such as transport, low temeporatures, bad microclimate, deficient nutrition, overcrowding in facilities, frequent exposure to stress factors. The consequence of non-infectious factors is the daily production of a large amount of gases, water vapor, heat and bio-aerosol particles, which directly affect the change in the physical and chemical composition of the air inside the facility (Stanković B. et.al. 2009, Bojkovski J., 2015b). A change in the physical and chemical composition of the air favors respiratory pathogens and their continuous maintenance of a high degree of virulence (Stanković B. et.al. 2008). Dust, which is usually found in large quantities in pig facilities, has a mechanical and chemical effect on the nasopharyngeal and bronchial ciliated epithelium. Due to the increased action of predisposing factors, the epithelium of the mucous membrane of the respiratory system is damaged, its activity decreases, and thus the possibility of continuous elimination of accumulated exudate and inhabited microorganisms (Lončarević et.al. 1997, Kureljušić B. et.al. 2016).As a result of the above-mentioned problems, the occurrence of respiratory infections is more frequent, the control of which is difficult. Technological systems that do not include the "all-in/all-out" procedure , and contain pigs from various locations, introduce gilts into breeding without prior health control and thus form groups of different immune status, which enable infecting the throat with numerous pathogens (Lončarević A. et.al.1997, Šamanc, H. 2009, Weissenbacher-Lang C.et.al.2016). Changes in environmental conditions lead to stress, which, like various infectious agents, can significantly suppress respiratory defense mechanisms in pigs. To that, we should definitely add the fact that the farms where we monitored the health status of the pigs rarely carry out the "facility rest" procedure, which would allow minimal exposure to endemic pathogens and thus lead to the development and uniformity of the immune status of all individuals in the group (Bojkovski J. et.al. 2005, Kureljušić B. et al. 2015).

An important factor in the pathogenesis of the disease is the susceptibility of the blood vessels of the lungs to the action of numerous immune processes. As a result, damage to the walls of blood vessels occurs, their permeability increases, and as a result, edema is created, which provides a suitable basis for the further pathogenic action of many agents (Šamanc,H. 2009). Due to the action of pathogenic microorganisms or their toxic products, the defensive activity in the lungs is impaired, and the circulation is particularly difficult, especially in the parenchyma around the edges of the lung lobes. In this, non-specific factors, such as microclimate and cold, are particularly important. It has been proven that peripheral cooling can cause disruption of blood flow through the lungs, with consequent changes in ciliary activity, mucus production, reduction of local cellular and immune defense activity (ŠamancH. 2009).

The pathomorphological changes that occur in respiratory infections are characteristic and depend on the type of infectious nose, as well as on the routes by which they reach the lungs (Jovanović M. et.al. 2019). The most common route of infection is aerogenous, so the causative agents reach the lungs through the bronchial tree, where they first spread endobronchially, and then secondarily through the lymphatics into the peribronchial spaces. The causative agents can reach the lung tissue and hematogenously, especially after septicemic conditions. First, they settle in the interalveolar, i.e. peribronchial space (Radojičić B. et.al. 2002, Lupulović D. et.al. 2008 ,Radojičić S. et.al. 2011). Regarding the spread of the causative agent and the origin of the infection aerogenously, there are different points of view. It is believed that nasal secretions, as infectious material, can reach the oral cavity and thus allow the causative agents to first settle in the tonsils and pharyngeal mucosa, and from there reach the respiratory tract (Šamanc, 2009, Lipej, 2015, Savić, 2016, Jovanović et al., 2019).

In this article, we wanted to give an overview of our research on respiratory diseases of viral etiology in pigs from commercial farms.

Reproductive-respiratory syndrome of pigs (PRRS)

RESULTS AND DISCUSSIONS

The PRRS virus acts on specific host cells, infecting only specific subsets of porcine macrophages (Šamanc H. 2009). This virus has a special affinity for alveolar macrophages and destroys up to 40% of these cells. The virus leads to the lysis of pulmonary alveolar macrophages and pulmonary intravascular monocytes, which represent the primary sites of virus replication in the lungs, there by further reducing the body's defense ability. The process of PRRS virus entry into macrophages has been described as pH-dependent, receptor-mediated endocytosis. In the first week after infection, there is a dramatic decline in lung alveolar macrophages, and their defensive function is severely impaired. Certain

studies also indicate a significant accelerated apoptosis of macrophages in lungs infected with the PRRS virus, which contributes much more to the decline in the number of macrophages if you take into account the fact that only 2% of lung macrophages are directly infected by the virus in the acute phase of inflammation. Damage to pulmonary alveolar macrophages and intravascular monocytes lead to increased susceptibility to bacterial infections from the development of severe pneumonia and septicemia (Šamanc H.2009). Direct contact is the most important way of transmitting the virus. The infection is peroral, aerogenous and coital (including artificial insemination). Pigs infected with the virus that causes PRRS most often exhibit symptoms that include inappetence, elevated body temperature, lethargy and difficulty breathing. Important factors that influence the nature, course and prevalence of the disease are the essence of the population, the quality of the ventilation systems on the farm, the mixing of production categories, the health status of the animals, the way they are kept, the amount and strain of the virus circulating in the group of animals (ŠamancH. 2009, Lipej Z.2009, Radojičić S. et.al., 2011, Bojkovski J.et. a.l 2012, 2015b, Savić B. et. al. 2016).

Sows infected from the 85th to the 90th day of pregnancy can farrow persistently infected piglets in which the RNA virus can be detected up to 210 days after parturition (Petrujikić et.al. 2011). In persistently infected piglets, mortality before farrowing is high and changes in respiratory they are difficult for the organs, and at the same time, such piglets are also a significant source of the dissemination of the virus and its persistence on the farm (Štukelj,M. 2017). More severe clinical symptoms and greater losses are recorded on large farms that do not quarantine newly acquired animals and where zoohygienic conditions are inadequate. However, the PRRS virus causes the most harmful effects in younger categories of piglets and sows. In gilt sows, PRRS infection causes partial placental detachment, which can lead to abortions, premature births, or death and mummification of the fetus. Abortions in late pregnancy were recorded in 30% of infected sows, with the number of stillborn piglets reaching 100%. Liveborn piglets infected prenatally are usually weakly vital and show severe respiratory symptoms, with up to 80% dying on a weekly basis before weaning, so it often happens that only one piglet per litter is weaned (Petrujkić T.et.al. 2011). Diarrhea and severe respiratory disorders caused by lung damage often occur in young piglets infected with the PRRS virus postnatally. In suckling piglets, the infection can be transmitted from infected sows via milk (Petrujkić T. et.al. 2011). At this age, the infection leads to a fatal outcome in as many as 80% of animals after confinement. The mortality rate is decreasing, but the economic losses caused by reduced daily gain and reduced food conversion continue (Petrujkić et al. 2011). A few days after the appearance of a high temperature, a cough begins, and redness and cyanotic changes appear on the skin of the neck, ears, back, peri-anal region and upper parts of the hind limbs. Infection in the middle of the gestation period can be accompanied by abortions, mummification of fetuses, early death of embryos and consequent sterility. A reliable sign of infection in the throat in reproduction is reflected in the reduction of the number of pollinated piglets and the irregular occurrence of estrus, but also as a transition to anorexia and early farrowing (Šamanc,H.2009, Petrujkić T.et.al 2011). In dead animals, pathomorphological changes in the respiratory tract may indicate the disease, but in PRRS there is no pathognomonic finding (ŠamancH. 2009). In newborn piglets and weaned piglets in fattening, moderate to severe, multifocal to diffuse lung changes occur. The most obvious macroscopic change is the enlargement of the lymph nodes, starting on the 10th day after virus inoculation. Microscopic findings on the lungs of young piglets affected by interstitial pneumonia are characterized by infiltration of alveolar septa with mononuclear cells, hypertrophy and hyperplasia of type II pneumocytes and alveolar exudate composed of edema fluid and mononuclear cells. Lymphohistiocytic encephalitis, myocarditis and rhinitis can occur in some cases (ŠamancH. 2009). Changes are equally present in the lung tissue of the apical, medial and caudal lobes. In the case of secondary bacterial infections, the main pathological changes may not be due to infection with the PRRS virus. Changes can be found in the blood vessels in the form of perivasculitis, myocarditis and splenitis with a decrease in the number of lymphocytes (Šamanc H. 2009). Today, PRRS occurs predominantly in Europe as a respiratory syndrome in the category of suckling piglets and in the period of early fattening (Bojkovski J. et.al. 2012 a,b, Štukelj,M. 2017, Obrenović S. et.al. 2019).

In Serbia, the disease was first described clinically in 1998 in pigs in Vojvodina, and during 2000 it was serologically confirmed. It is considered that PRRS is an underestimated and uncontrolled respiratory swine disease in Serbia. According to Petrović T. et al. (2011) the first suspected cases of PRRS in Serbia occurred in 2001, when serious respiratory disorders associated with high mortality affected pigs on two industrial

farms located in the Northern region close to the borders with Croatia and Hungary. The suspected cause of the cases was boar semen illegally imported from neighboring countries. Subsequently in 2001-2002, respiratory syndrome with high morbidity and moderate mortality, which was diagnosed as PRRS, occurred on several commercial farms in the Northern Serbian province of Vojvodina and later on in the central part of Serbia (PetrovićT. et.al 2011, Prodanov-Rsadulović, J.et.al. 2020). Severe health problems and high economic losses led the Veterinary Directorate to perform PRRS serology screening in 2002, 2004-2005 and 2006-2007. In the majority of the studied herds, PRRSV antibodies were detected in a very high proportion of fatteners. Monitoring in 2006-2007 revealed PRRSV-positive herds in all Serbian regions at prevalence of 1.56-60.86%. No other monitoring or control program against PRRS was proposed at the national level. However, as a consequence of the first virus introduction and resulting outbreak, an emergency PRRS vaccination campaign was carried out on a small number of commercial farms in Northern Serbia in 2002-2003. The obtained results in the last 15 years suggest that PRRS virus infection is widely distributed in Serbia. Phylogenetic analysis revealed that all genetically typed isolates belong to the EU subtype 1 or Lelystad type viruses that are distributed globally in Europe, as well as in the other parts of the world. This result was expected regarding the results published from surrounding and other EU countries.(Novosel D. et al., 2016). Despite the fact that the disease is widespread in most of the commercial swine farms, there are no legislation procedures regarding PRRSV control in Serbia. To the best of our knowledge, today the vaccination against PRRS virus is used in a number of Serbian commercial pig farms. Also, at the moment PRRS monitoring and surveillance are not undertaken except for the animals imported from another country and in abortion cases that are sent to laboratory testing. However, it needs to be stressed that Serbia as a Western Balkan country annually imports a large number of different categories of live pigs from Western Europe. The preventive measures are only done through serological testing of breeding animals (gilts, sows, boars), farm management and biosecurity protocols.(Prodanov-Radulović J. et al., 2020)

In North Macedonia porcine reproductive and respiratory syndrome virus in was first laboratory detected in 2015 after an acute outbreak of respiratory disease in one pig farm with 650 sows. Twenty animals including weaned and grower pigs were bled and samples were analyzed for PRRSV. After laboratory confirmation on PCR, farm has started vaccinating against PRRSV. Later in 2016, another commercial pig farm with 500 sows in southern region of the country reported clinical outbreak of abortions in sows. After consultation with the Faculty of veterinary medicine in Skopje (FVMS), farm has brought sera samples and vaginal swabs from aborted sows. Samples were tested on PCR and PRRSV was diagnosed. Introducing of the virus in these farms probably has occurred due to importing new gilts for replacement of breeding animals. Additionally, these two farms shared same truck for animal transport from abroad and that was the most likely way of PRRSV introduction into herds. In same 2016, small-scale pig farm complained about poor growth rate, dyspnea, rough hair coat and dehydration in weaning pigs. Farm was visited and blood samples were taken from pigs at 8 to 12 weeks of age. All samples were tested on ELISA and PRRSV antibodies were detected. In 2018, two commercial pig farms with one-site production system (150 to 170 sows) located in the central part of the country had experience with growth retardation and severe respiratory clinical signs. We visited these two farms and necropsy was performed on several carcasses from pigs at 10 to 12 weeks of age. The lesions were suggestive of PRRSV-like induced interstitial pneumonia. Blood samples from weaned pigs were taken in order to be tested on PRRSV. Samples were positive on both ELISA and PCR and mass herd vaccination was recommended.In the same year, small farm with 50 sows located in the west part of the country contacted FVMS and asked for an expert opinion related with poor growth rate, rough hair coat, anorexia and dyspnea in weaned pigs. On the day of farm visit, we take blood samples from several pigs with clinical respiratory sings. Samples were tested on PCR and gave positive results on PRRSV. The PRRSV in this farm was introduced most probably via breeding gilts which were purchased from a farm that vaccinates against PRSSV. Recently in 2021, at request of a farm with 170 sows, 50 blood samples from different category were taken and tested o determine PRRSV circulation. All samples were positive on ELISA and negative on RT-PCR indicating that the farm infection has occurred earlier and the herd has established collective immunity. Very recently in December 2021, large pig farm reported late abortions in sows and cyanosis of ears and skin in finishing pigs. Blood samples from aborted sows and pigs with cyanosis were tested for PRRSV. Samples were analyzed and gave negative results on PCR. However, positive samples on ELISA indicating that the sampled animals most likely had

been infected earlier and developed neutralizing antibodies. According to obtained results, PRRSV is probably endemic in most of the commercial pig farms in North Macedonia. Unfortunately, there is no national program for monitoring and surveillance of PRRSV either for commercial pig farms or for imported pigs. National strategy is needed for control and monitoring of PRRSV due to great economic losses that could affect the pig industry. All obtained results regarding PRRSV in North Macedonia are personal data and are not published yet (Angjelovski).

Swine influence

Swine influenza is a highly contagious, acute viral disease of the respiratory system of pigs, present in many countries with developed pig farming (ŠamncH. 2009, Lipej Z. 2015). It is characterized by respiratory disturbances in the form of catarrhal inflammation of the respiratory system, difficult and irregular breathing and strong coughing. The disease appears suddenly with symptoms of elevated body temperature, anorexia, prostration and dyspnea. Morbidity reaches 100%, and sick pigs usually recover quickly. In such cases, mortality is less than 1%. The economic consequences of influenza are significant. Growth is reduced, and deaths are most often the result of secondary bacterial infections (Lončarević A.et al. 1997, ŠamancH. 2009, Maksimović Zorić J et al. 2017).

The three basic subtypes of influenza A virus circulating in pigs are H1N1, H3N2 and H1N2. The most common way of transmitting the virus between a sick and susceptible animal is through aerosols, droplets and feces, and the fastest way of spreading the virus is through nasopharyngeal secretions. (Lončarević et al. 1997, Šamanc, 2009).
After reaching the body of a susceptible individual, the virus is accepted by the cilia of the mucous membrane epithelium of the respiratory system (nose, trachea and bronchi) and after initial replication in individual cells, within 1 to 3 days, it spreads throughout the respiratory system. During the appearance of the most pronounced clinical symptoms, necrosis is emphasized and practically all the cells of the bronchial mucosa are affected. (Lončarević A., et al. 1997, Šamanc H., 2009) At that time, the dominant findings were lobular atelectasis, emphysema, and focal coagulation necrosis of the bronchial epithelium. Within 72 hours, the pathological process reaches its maximum, after which the multiplication of the virus decreases, and the symptoms subside. Bacterial infections, such as *Haemophilus parasuis* and *Bordetella bronchiseptica*, play an important role in the development of the pathological process (LončarevićA. et al. 1997, Šamanc, 2009). In all or most animals in the zoo, clinical symptoms appear suddenly within 1 to 3 days of infection. Prostration, apathy and anorexia are the first symptoms.
Body temperature is elevated. Cough and shortness of breath occur. In addition, rhinitis, nasal discharge, lacrimation and conjunctivitis may occur. Weakness of muscles, loss of body mass is observed, and after being sick for 3 to 6 days, the animal starts to recover. As a rule, all pigs in the barn start eating normally around the seventh day after the appearance of the first symptoms of the disease. Subclinical infections are also possible (Lončarević A. et al. 1997, ŠamancH. 2009). Before and during gestation, infection can cause reproductive problems: infertility, fetal death, abortion, small litters and farrowing of weak piglets. However, in this case it is not a direct effect of the virus, but symptoms that can disrupt the reproductive characteristics of the sow. (Lončarević A. et al. 1997, Šamanc H. 2009).

Macroscopic changes are localized in the lungs, most often in the apical and cardiac lobes. Parts of the lung are consolidated, dark red and purple in color and are clearly demarcated from normal tissue. Hyperemia and the presence of exudate are emphasized. Bronchus and bronchioles are dilated and filled with exudate. Also, in more severe cases, multifocal to diffuse pneumonia may develop with dark red to yellow-brown fields that can affect from 20% to 100% of lung tissue. Hyperemic tracheobronchial and mediastinal lymph nodes are often enlarged. (Lončarević A.et al. 1997, Šamanc, H.2009, Jovanović M. et al. 2019).

Pathohistologically, there are diffuse degenerative changes and necrosis of the epithelium of the bronchi and bronchioles. The lumen of the bronchi, bronchioles and alveoli is filled with exudate containing desquamated cells and neutrophilic granulocytes. Microscopic findings of lung tissue are characterized by broncho-interstitial pneumonia with necrotic bronchitis and bronchiolitis, infiltration of alveolar septa by inflammatory cells, hypertrophy and hyperplasia of type II pneumocytes, and filling of airways and alveolar spaces with liquid containing proteins and various inflammatory cells (Lončarević A.et.al. 1997, Šamanc,H. 2009, Jovanović M. et al. 2019). Although , swine influenza virus is often isolated from individuals suffering from respiratory diseases, and seroconversion has often been demonstrated in growing and fattening pigs, its role in the

pathogenesis of diseases from the respiratory complex is still not entirely clear. By damaging the mucociliary apparatus and reducing the function of macrophages, the virus can lead to an increase in the predisposition of pigs to bacterial pneumonia. Also, in combination with other respiratory viruses, swine influenza virus can cause much more severe pathomorphological changes, which will be manifested by more severe clinical symptoms and increased mortality. It has been proven that the influenza virus in co-infection with the PRRS virus or *M. Hyopneumoniae* causes severe morphological changes and prolongs the duration of respiratory diseases in pigs (Lončarević A.et.al. 1997, Šamanc,H.2009, Jovanović M et.al. 2019). Contrary to this, in co-infection with circovirus, influenza virus is isolated much less

Circovirus infection of pigs

Porcine circovirus type 2 (PCV2), from an economic point of view, is one of the most important causative agents of swine diseases. In the group of circovirus diseases, in addition to PMWS, there are also disorders in reproduction, dermatitis - nephropathy syndrome (Porcine dermatitis nephropathy syndrome - PDNS,), as well as respiratory and enteric forms of this disease. Today, these pathological entities are called porcine circovirus associated diseases (PCVAD) by one name (Savić B.et al. 2012a). Based on new knowledge about the pathology of this disease, a new terminology was proposed, the subclinical forms of circovirus infection known today in veterinary practice, then PCV2 systemic disease (Porcine circovirus systemic disease; PCV-2-SD,), respiratory form of PCV2 disease (Porcine circo virus lung disease; PCV- 2-LD), enteric form (Porcine circo virus enteric disease; PCV2-ED), increasingly common reproductive form (Porcine circo virus reproductive diseases; PCV2-RD) and dermatitis nephropathy syndrome (Savić B.et.al. 2012 b).The source of infection is mostly sick animals, and the clinical manifestations depend on the affected organ system. It is not possible to predict which of the systems will be affected, and therefore which manifest form will appear Any manifest form that is involved, damages, both direct and indirect, are always very significant and threaten any rational production of pigs. Of all these, PMWS is the most economically significant disease. This syndrome, as one of the forms of circovirus infections, is present in the pig population in the Republic of Serbia and occurs in pigs between 6 and 16 weeks of age (Ivetić V. et.al., 2004).

Pig circovirus 2 belongs to the genus Circoviruses and causes diseases in pigs, and was isolated in cases of multisystemic stunting syndrome in piglets, followed by the development of porcine dermatitis and nephritis syndrome, i.e. in pigs with reproductive disorders. Three genotypes of the mentioned virus have been described: PCV-2a, PCV-2b and PCV-2c (Ivetić V.et al. 2004., 2005).
The oronasal route is considered the most likely and most common route of transmission of PCV2 infections. This claim has been proven by a large number of experimental studies on circovirus infections, which mostly used the intranasal route of inoculation of this virus (Lipej Z., 2015). Although, the oronasal route is considered the most common, the PCV2 virus can also be transmitted by almost all secretions and excreta, tonsillar, bronchial, eye secretions, as well as feces, saliva, urine and sperm (Ivetić, 2005). The presence of PCV2 was also found in colostrum (Petrujkić, 2011), but whether such a finding can lead to infection is still unknown (Petrujkić T.et.al 2011). The pathogenesis of circovirus infections and the cell types that support PCV2 replication are still not fully understood. Lymphocyte depletion and lymphopenia in the peripheral blood are a constant finding in piglets with a developed clinical picture of PCVAD (Šamanc, 2009).

Piglet multisystem wasting syndrome most often occurs in piglets between the ages of 2 and 4 months, although the disease can occur in pigs between the ages of 1 and 6 months. This syndrome is present in almost all types of farms with a capacity of 30 to 10,000 sows. Morbidity and mortality on farms where PMWS occurs is variable, depending on housing, management, co-infections and other factors related to pig production. Morbidity ranges between 3-40% (extremely rarely over 50%) and mortality on affected farms is between 4-20% (IvetićV. et.al. 2004).

The main clinical sign of PMWS is loss of body weight, and often along with this symptom, there are other signs, such as pale skin, difficulty breathing, diarrhea, swelling of the eyelids, and occasionally icterus. Piglets have coarse hair, take a characteristic "buried" or "thoughtful" position with their head down (Ivetić V.et.al.., 2004). Stunted piglets can not recover and have to be removed from the pen due to significant cachexia. A prominent feature in pigs in the early clinical phase of PMWS is an increase in subcutaneous lymph nodes, most often inguinal superficial lymph nodes, although infections are possible without this symptom. Neurological symptoms are observed less often (Ivetić V.et.al. 2004).

On the section of dead piglets, lesions on the lungs and enlargement of lymph nodes (inguinal, sub mandibular, mesenteric and mediastinal) are most often found. The superficial inguinal lymph nodes are most often affected, which on cross-section show a red-brown zone interspersed with bacon fields. In addition to the inguinal, the enteric lymph nodes stand out for their size, so that in some cases they take on the size of a child's forearm. However, these lesions are not always present, so they can not serve as markers for PMWS on pig farm . In a certain number of piglets, necrotic changes in the liver with noticeable discoloration are observed, with jaundice evident. Also, with the piglet, let's take care of the multifocal leaflets in the kidneys. A smaller number of piglets suffering from PMWS may have bronchopneumonia and gastric ulceration in the esophageal serosa that are not directly related to circovirus, but more to secondary infections. These lesions cause internal bleeding that can lead to death in piglets with PMWS and are responsible for pale skin. At the end of this stage, cachexia may develop (Ivetić,V. 2004, Jovanović M. et.al.2019).

Characteristic microscopic lesions can be found in the lymph nodes. These are clearly circumscribed, spherical, basophilic, cytoplasmic inclusions of PCV2 in histiocytes. In the portal zone of the liver, a lymphocytic-histiocytic inflammatory infiltrate, necrosis of individual hepatocytes, etc. can be observed. However, in some cases, generalized perifocal necrosis with massive loss of hepatocytes is observed. Other microscopic changes of a similar nature can be observed in the kidneys, pancreas, intestines and myocardium. Moderate to severe granulomatous enteritis also occurs sporadically (Ivetić V.et al., 2004). In piglets suffering from PDNS, anorexia, depression, loss of consciousness, unsteady gait occur, with individuals having a physiological temperature or mild pyrexia. However, the most obvious sign in the acute phase of the disease is the presence of irregular, red papules and macules on the skin, mainly on the hind limbs and perineal region, which tend to coalesce. Over time, the lesions become covered with dark scabs and gradually fade, some times leaving scars. The cause of death in pigs affected by PDNS is acute renal failure, with usually a very significant increase in serum urea and creatinine (Ivetić V.et.al 2004).

In addition to skin changes, in pigs that die from an acute infection of PDNS, the kidneys are enlarged with numerous cortical hemorrhages and edema of the renal pelvis (Ivetić et.al 2004, Jovanović, 2019). Histologically, non-purulent interstitial nephritis with dilatation of renal tubules can be observed. Kidney and skin lesions are most often present in pigs suffering from PDNS, but there are also rare cases when skin or kidney lesions can occur by themselves (Ivetić et.al. 2004). Renal lymph nodes, as well as other lymph nodes, can be enlarged and red in color due to blood drainage from the affected areas, mainly the skin (Ivetić V.et.al 2004).
In the respiratory form of circovirus disease, the main clinical signs are respiratory disorders and diarrhea (Ivetić 2004). Given that these clinical signs are also present in PMWS, there may potentially be a diagnostic overlap between these forms of circovirus infection (Došen R. et.al. 2005).

Before commercial vaccines became available, successful treatment and control of PCVAD was primarily focused on ensuring good manufacturing practices that minimize stress, eliminate or reduce co-infections, and eliminate potential factors that induce immune stimulation and progression of PCV2 infections. Today, Madek's 20-point plan for controlling PCV2 infections is used, which can be summarized in 4 golden rules, which include: 1) limiting contact with pigs, 2) reducing stress, 3) good hygiene, 4) good nutrition. One of the main points of Madek's plan is to minimize contact between pigs, given that direct contact is one of the most common ways of spreading this infection in the herd. It is recommended to establish solid partitions between boxes, as well as to adopt the "all in, all out" system on farms in order to reduce contact between pigs. The quarantine of newly acquired pigs aims to prevent the introduction of new infections. An important element in the control of PCV2 infections is the suppression of certain diseases such as PPV, PRRS, enzootic pneumonia and swine flu, which increase the severity of PCV2-induced lesions. (Madec F. et al., 1999)

Respiratory form of swine corona virus disease

When we talk about corona virus diseases of pigs, we primarily mean diseases of the digestive system in the form of transmissible gastroenteritis of pigs (Transmissible gastroenteritis - TGE). In general, porcine corona viruses cause vomiting and weight loss in pigs, epizootic diarrhea in piglets, transmissible gastroenteritis and respiratory disease. The porcine respiratory corona virus (PRCV) is only one form of the virus that causes TGE with a changed tropism from the intestinal to the respiratory system. The main difference between the corona virus that causes TGE and the virus that causes respiratory disorders is the

deletion in the S gene that exists in the respiratory form of the swine corona virus (Šamanc H, 2009). The change in the antigenic composition, which explains the appearance of the virus that primarily leads to respiratory disorders in pigs, may also be a consequence of the TGE vaccination program (Šamanc H, 2009).

An important mechanism of virus acceptance for the receptive cell is the interaction of the viral S glycoprotein with receptors on the cell surface. These receptors are also glycoprotein in nature and are characteristic of each receptive species. The entire process of viral replication takes place in the cytoplasm. Moreover, this virus can multiply in cells without a nucleus, which is important for the pathogenesis of some diseases caused by corona viruses. The mutant virus that causes changes in the intestines, as most mentioned, can cause I respiratory problems, and it differs only in the E2 viral protein, which is a consequence of the mutation in the S gene (Šamanc H, 2009).

The significance of respiratory corona viruses is manifold. Certain isolates can induce or contribute to the development of viral diseases. Serologically, it is difficult to distinguish respiratory and enteral forms of corona virus. Respiratory corona viruses can induce cross-protection against enteric corona viruses (Šamanc, 2009).

A susceptible animal can become infected by ingesting or inhaling contaminated material. The infectious dose is small and depends on the age of the animal. In the pig population, the disease spreads aerogenically and through contaminated feed. Within a farm and between farms in one region, the disease can also be transmitted by secondary sources of infection, which include means of transport, shoes and clothing of people, such as by air, up to several kilometers. Non-specific factors play a significant role in the development of the disease, especially cold weather, improper diet, frozen or spoiled fodder and other factors that can reduce the animal's resistance. The respiratory form of the disease begins with the primary multiplication of the virus in the mucous membrane of the nose, trachea and lungs. After the primary replication of the virus in the epithelium of the initial parts of the respiratory tract, the virus invades the bronchial and bronchiolar epithelia and further spreads to the peribronchiolar and alveolar spaces. By passage through a greater number of hosts, the virus becomes more pathogenic.

Pigs can be positive for the respiratory corona virus without showing symptoms characteristic of diseases of the respiratory tract. Clinical symptoms are non-specific and manifest as varying degrees of anorexia, lethargy, elevated temperature, difficult and accelerated breathing, weight loss, and in some cases end in death (Šamanc H, 2009). The carcasses of dead piglets are mostly cachectic and dehydrated. Macroscopic lesions, as well as clinical symptoms, vary from inapparent, difficult to detect, to multifocal changes on the lungs, which can progress to complete lung consolidation. A microscopic examination of the changed parts of the lungs reveals a bronchointerstitial pneumonia, characterized by necrosis, metaplasia and proliferation of the bronchiolar epithelium, as well as an increase in the number of neutrophil granulocytes and macrophages in the alveolar spaces (Šamanc H, 2009).

By weakening the defensive function of pulmonary alveolar macrophages, corona viruses lead to a weakening of the overall defensive ability of the respiratory system of pigs. Although, the role of this virus in the complex of swine respiratory diseases is still not entirely clear, it is not unusual for the virus to be isolated together with the PRRS virus and/or the swine influenza virus, all of which can contribute to the faster development of secondary bacterial infections (Šamanc H, 2009).

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ON-FARM MILK CULTURING FOR BOVINE MASTITIS MANAGEMENT

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Abstract

Bovine mastitis is the most common reason for antibiotic usage in dairy cows, its costs being estimated at more than $350 per mastitis case. Accurately identifying the bacterial species at the core of a mastitis infection may help narrow down treatment choices and cut down on the overuse of antibiotics. The aim of the present study was to evaluate the prevalence of etiological agents in milk samples from cows with clinical mastitis using commercially available culture plates (*ClearMilk*, LabMediaServis, Czech Republic). Briefly, milk samples were collected from udder quarters with clinical mastitis and were aerobically cultured using the on-farm culture system. If two prominent mastitis pathogens were identified, the sample was labelled as "mixed growth," and if three or more pathogens were observed, it was labelled as "contaminated". Our results indicate that ClearMilk might be an useful on-farm milk culturing tool for detecting pathogens linked with bovine mastitis.

Key words: mastitis; on-farm culturing; milk; antibiotics

INTRODUCTION

The most common reason for antibiotic usage in dairy cows is mastitis. Clinical mastitis may result in annual costs of more than $350 per cow due to treatment and wasted milk, according to one research. Clinical mastitis is often treated with antibiotics, despite the fact that drugs are often inefficient or unnecessary in treating the condition (Dego, 2020).

The most prevalent mastitis pathogens are found in udder tissues (contagious pathogens such as *Staphylococcus aureus*, *Streptococcus uberis* or *Escherichia coli*) or in the herd's environment (environmental pathogens), such as bedding, manure, and soil (Heikkilä *et al.*, 2018).

Accurately identifying the bacterial species at the core of a mastitis infection may help narrow down treatment choices and cut down on the overuse of antibiotics. Adapting herd management practices may reduce the prevalence of some pathogens. During milking, the parlor might get contaminated with pathogens like Staphylococcus aureus. Milking affected cows last, dipping teats, and using gloves are all strategies to limit the spread of the disease. If the culprit pathogen could be identified, farmers may adapt their practices to lessen the likelihood of infecting any further cows. A farmer's decision to not treat a cow may also be influenced by local norms.

In order to identify the pathogen agent, producers may submit milk samples to nearby labs for culturing. The delay of several days between milk submission and farmers receiving test results is the major drawback of this conventional diagnosis method. Since producers have to wait for test findings, they often make uninformed treatment choices. Thus, timely preventative treatment choices may be implemented with the support of an on-farm culturing system (Ganda *et al*, 2016).

The aim of the present study was to evaluate the prevalence of etiological agents in milk samples from cows with clinical mastitis using commercially available culture plates (ClearMilk, LabMediaServis, Czech Republic).

MATERIAL AND METHOD

Milk sample collection

The study was conducted to evaluate the efficiency of on-farm culture system in identifying bacteria linked with clinical mastitis, using 29 milk samples collected within a four-month interval between July and October of 2022, from the dairy farm within the research center. Milk samples were collected aseptically by qualified research employees from mammary quarters with clinical mastitis using a methodology previously described by Ferreira, *et al*. 2018.

Teats were cleansed and disinfected with a napkin containing 70% ethanol. The first strips

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were discharged before milk samples were collected in sterile 12 mL tubes, without preservatives.

Clinical assessment

A clinical mastitis case was defined as the presence of abnormal milk (i.e., watery secretion, occurrence of flakes or clots) with or without visible signs udder or general inflammation (i.e., swelling, redness, pain, increased local heat, fever, milk yield decrease), which were detected by trained research personnel at each milking.

The severity of the clinical infection was assessed by trained research personnel as described previously by Bradley and Green, 2001. Clinical mastitis score 1 was attributed to cases in which macroscopic modifications in milk were observed, but no other inflammation signs were present. Clinical cases in which both macroscopic modifications in milk and udder inflammation signs were present were considered as score 2. Finally, cases in which the macroscopic modification of milk were present alongside udder inflammation signs and general inflammation sign such as fever were considered as score 3.

The herd samples were taken to the Research and Development Station for Cattle Breeding Dancu, Iasi, Laboratory of Nutrition, Food Quality and Safety where they analyzed withing two hours from sample collection.

On-farm culturing test

The inoculation wand included in the kit was dipped in the sterile plastic tube holding the milk sample, and then wiped over the surface of each Petri dish with selected growth medium in a zig-zag pattern in accordance with manufacturer's specifications. Plates were incubated aerobically at 37°C for 24 hours before being read on-site by a single member of the research team.

Interpretation of the culturing results

If two prominent mastitis pathogens were identified, the sample was labelled as "mixed growth," and if three or more pathogens were observed, it was labelled as "contaminated". The following were the preliminary selection criteria for microorganisms: five identical colonies were found for major pathogens (with the exception of S. aureus and Streptococcus, where the presence of a single colony was deemed positive); 10 identical colonies for the other bacteria.

RESULTS AND DISCUSSIONS

Figure 1 depicts the percentage of mastitis caused by various infections on each farm. The most frequent encountered pathogen in the collected milk sample was *Streptococcus dysgalactia* (24.14 % of the cases). *Streptococcus spp.* are a prominent cause of bovine mastitis across the globe, impacting both milk production and animal welfare. Pathogens *Streptococcus dysgalactia* or *Streptococcus uberis* are often found in manure and organic material, including bedding materials, and may colonize both animals and the environment, potentially leading to both clinical and sub-clinical mastitis in dairy cattle (Wente *et al.*, 2019).

Dairy cows are most typically infected through environmental sources during the dry period, often leading to clinical cases during the subsequent lactation. In our study, 75% of the cows diagnosed with clinical mastitis, in which *Streptococcus uberis* was identified as causative agent, presented both macroscopic modifications in milk as well as local signs of infections, consistent with a mastitis score 2, while 25% additionally presented an overall altered health status, consistent with mastitis score 3. Another major pathogen that generated more severe infection manifestation is *Escherichia coli* (Figure 1, Table 1). Milk modifications and udder inflammation signs are generally accompanied by acute general symptoms such as diarrhoea, and chilled extremities. Because this pathogen may cause serious foodborne illness, its presence is considered a food safety concern and a health risk for both people and animals (Goulart and Mellata, 2022).

0ver 17% of the analyzed milk samples were positive for Coagulase-negative staphylococci (CoNS), *Staphylococcus chromogenes* being the main coagulase-negative staphylococci isolated from the analyzed mastitis samples (Figure 1; Table 1). Coagulase-negative staphylococci (CoNS) are less virulent because they do not generate a high number of harmful enzymes and toxins (Lee and Lee, 2022). Yet, they are becoming more recognized as a significant cause of bovine mastitis due to their capacity to build biofilms which in turn may lead to increased resistance to antimicrobials.

The severity degree for the other pathogens was assessed as score 1, respectively score 2. This difference may be relevant when analyzing a herd's problems and the strategies that may be implemented to minimize or treat mastitis (Lee and Lee, 2022).

Even if the sampling collection protocol is accurately respected, there is still a risk of sample contamination (Figure 1), which is way the interpretation of the results should be made with carefully consideration. If a strongly changed inflammatory secretion is observed, treatment

options should be considered even if no growth was observed. Mastitis control has gained increasing attention as a result of the emphasis on the judicious use of antibiotics in dairy herds, especially in terms of prevention of new infections and alternative treatment approaches (Neculai-Valeanu *et al.*, 2021).

Concerns about antibiotic residues in farm-raised meat and milk were usually at the forefront of discussions about antibiotic stewardship in the dairy sector in order to guarantee a secure food supply. However, in the past years, antibiotic stewardship measures, which aim at reducing antibiotic-resistant bacteria to benefit both human and animal health, have become a key point of focus worldwide (More *et al.*, 2022).

While *Streptococcus uberis* requires extended parenteral and/or intramammary antibiotic treatment durations for at least 5 days, mastitis generated by coagulase-negative staphylococci may be treated exclusively with anti-inflammatory therapy (Smulski *et al.*, 2020). Mixed growth samples and no growth cases should be treated taking into consideration the presence or absence of macroscopic modifications in milk.

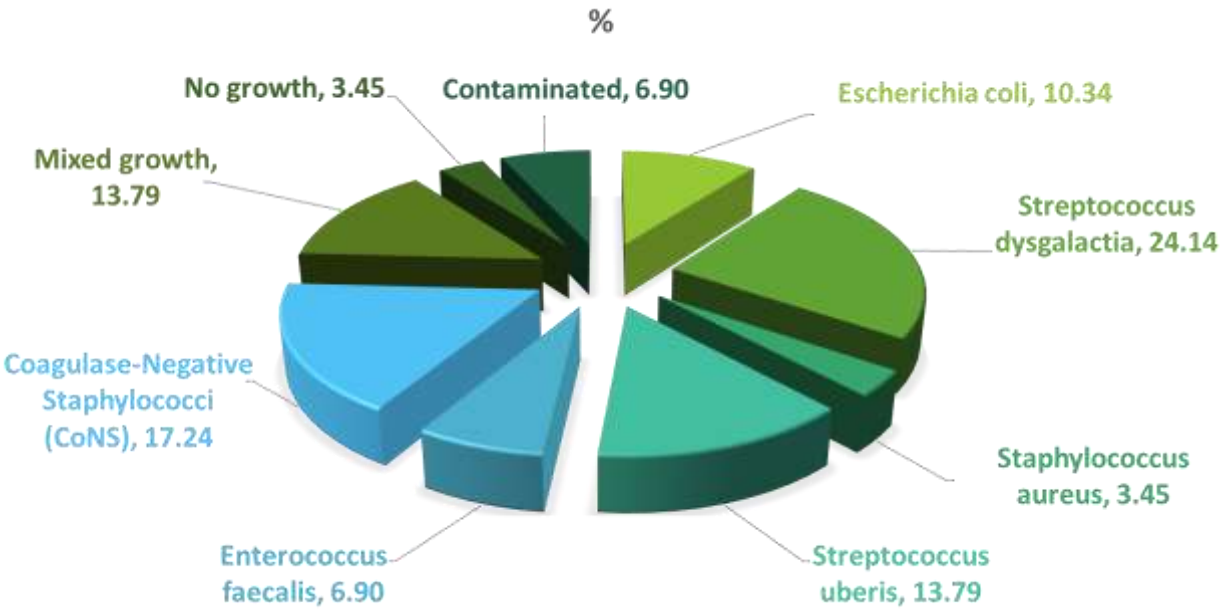

Figure 1 **Percentage distribution of mastitis pathogens in clinical mastitis samples with positive bacteriologic growth**

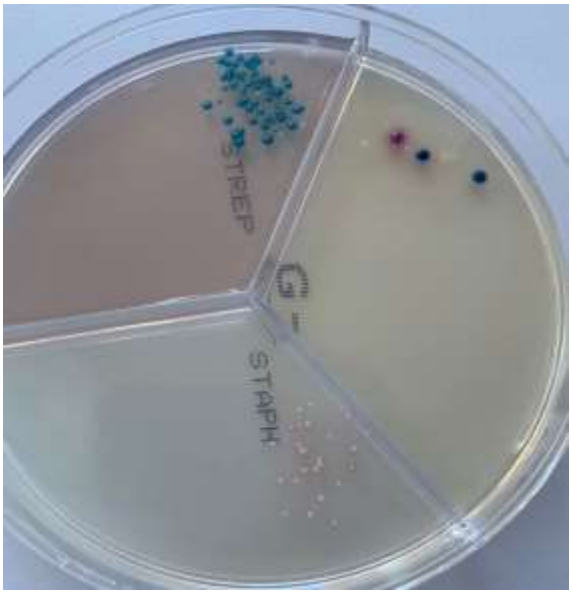

Figure 2 **Contaminated milk samples from cows with mastitis (*Streptococcus dysgalactia*, *coagulase-negative staphylococci* and *E. coli* colonies present in the sample**

Table 1

Percentage cases of mastitis of different grades of severity associated with each pathogen

Pathogen	Mastitis Score		
	1	2	3
Escherichia coli	-	33.33	66.67
Streptococcus uberis	71.43	28.57	-
Staphylococcus aureus	-	-	100
Streptococcus dysgalactiae	-	75.00	25.00
Enterococcus faecalis	50.00	50.00	-
Coagulase-Negative Staphylococci (CoNS)	60.00	40.00	-
Mixed growth	50.00	50.00	-
Contaminated	100.00	-	-

CONCLUSIONS

On farm culturing systems are intended to inform and guide farmers toward preventative mastitis treatment strategies. While antibiotics may not be considered necessary in mild clinical cases, pathogens such as *Streptococcus spp.* and *Staphylococcus aureus* should be handled with intramammary antibiotics to maximize cure and reduce risk of transmission. In fungal mastitis immunomodulators and modification of milking routine, with more milkings per day may improve the outcome. Our results indicate that ClearMilk might be an useful on-farm milk culturing tool for detecting pathogens linked with bovine mastitis in order to adopt an appropriate treatment protocol.

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MONITORING, PREVENTION AND TREATMENT AS KEY PILLARS IN THE MANAGEMENT OF BOVINE MASTITIS

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Abstract

The quality of milk is a component based on certain individual indicators that allow the correct assessment of the quality of the dairy product starting from the producing animal, the quality of the feed, the exploitation system, the state of health, milking hygiene, the storing and transportation conditions of milk towards the processing unit. The purpose of this study was to monitor udder health and establish a proper treatment based on a specialized protocol that employs the assessment of physicochemical milk parameters, number of somatic cells, as well as identification of the pathogen agent generating the disease. Milk samples from 69 dairy cows were collected at the beginning and middle of lactation, a total number of 19 cases of clinical and subclinical mastitis cases being observed. The physicochemical parameters of milk, somatic cell count, and pathogen prevalence were assessed. A very small significant negative relationship was observed between somatic cell score and lactose percentage, ($r = 0.667$, $p < .001$). The most frequent encountered pathogens were *Streptococcus spp*. A management approach for mastitis, as well as other diseases, should include the following major components: prevention, monitoring, and diagnosis.

Key words: bovine mastitis; somatic cells count; on-farm milk culturing; milk quality

Introduction

A fundamental problem of the twenty-first century will be to ensure a sustainable and secure supply of food to the world's rising population. As a result, the fundamental goal of dairy farming is to reduce milk losses and other losses associated with unhealthy cows. Furthermore, a move from therapeutic to preventative health management for mastitis and a broad range of other infectious and non-infectious bovine illnesses, some of which are known to have significant negative impacts on cow performance and well-being, would be preferred (Farschtschi *et al.*, 2022).

Bovine mastitis is caused by a variety of factors, including infectious pathogens, inadequate nutrition, and ineffective farm management (1, 2). It has an impact on animal welfare and causes huge economic losses owing to lower milk yield, decreased milk quality, higher treatment expenses, and early culling (Ajose *et al.*, 2022).

Milk quality is a component based on certain individual indicators that allow the correct assessment of the quality of the dairy product starting from the producing animal, the quality of the feed, the exploitation system, the state of health, milking hygiene, the storing and transportation conditions of milk towards the processing unit (Leitner *et al.*, 2019).

According to the EU standards, the overall quality of milk refers to the three pillars: compositional, nutritional and biological quality (milk composition, cryogenic point, milk density, milk acidity, presence/absence of antibiotics and inhibitory substances), hygienic quality (<100,000 NTG/ml - the total number of aerobic mesophilic germs/ml milk) and sanitary quality (<400,000 NCS/ml - the number of somatic cells/ml milk). The discharge of somatic cells in milk is the best indicator of the beginning of inflammation in the mammary gland, and it is the most extensively used trustworthy biomarker of udder health (Jadhav *et al*., 2018).

The purpose of this study was to monitor udder health and establish a proper treatment based on a specialized protocol that employs the assessment of Physico-chemical milk parameters, number of somatic cells, as well as identification of the pathogen agent generating the disease.

MATERIAL AND METHOD

The research was carried out in the laboratory and zootechnical biobase within the Research and Development Station for Cattle Breeding Dancu, Iasi, from March to September 2022. A total number of 69 cows were included in the study, from second to forth lactation were included in the study.

Milk sample collection

Milk sample collection was carried out in accordance with the provisions of National Veterinary Sanitary and Food Safety Authority Order no. 35 of 2016, milk samples being collected from cows at the beginning (more than 45 days) or

at mid lactation, being well known that in the last part of lactation, the number of somatic cells is physiologically higher. For the physico-chemical and microbiological analysis, milk samples were collected in a sterile container, refrigerated and transported in short time to the laboratory.

Determination of somatic cells count and physicho-chemical parameters

To determine the number of somatic cells and the physicho-chemical parameters, the milk samples collected from the cows included in the study were warmed up to 37^{O}C. Three determinations were made for each milk sample. The following physico-chemical parameters were assessed: fat, dry matter, protein, lactose, casein and density. The number of somatic cells was assessed according to the fluoro-opto-electronic method (SR EN ISO 13366-2:2007), using the CombiScope 600/300 FTIR System, Delta Instruments.

Identification of affected udder quarters

To identify udder quarters with mastitis, a topical-glandular approach was used based on the assessment of somatic cell count using the "cow side" method, California Mastitest. Briefly, following teat disinfection, the first strips of milk were discharged and a small amount of milk, from each quarter, was collected in the plastic pallet with 4 cups marked A, B, C and D. An equal amount of reagent was added to this milk sample. The two elements were homogenized by rotating movements and the results were read after 10 seconds.

On-farm milk culturing

For the individual milk samples with somatic cells values above 200.000 cells/ml, with or without visible signs of clinical mastitis, a microbiological evaluation was carried out to identify the potential pathogenic agent, using the ClearMilk kit. The milk sample was placed on the surface of the Petri dish using the inoculation wand and incubated for 22-26 hours at 37.5°C. The Atlas of Agents was used to identify the pathogen. The presence of one colony for major pathogen agents such as *E. coli*, and *Staphyloccocus aureus* was considered positive. For other major pathogens such as *Streptococcus spp.* five or more colonies were considered as positive. The samples in which more than two pathogens were identified were discharged and considered as contaminated.

Statistical analysis

The statistical analysis was carried out using Graphpad software. The mean and standard deviation for all physico-chemical parameters were determined to evaluate possible differences between healthy cows and those with mastitis. To create a normal distribution, milk somatic cell count (SCC) was converted to somatic cell score (SCS) (SCS = (log2 (SCC /100000) + 3) (Antanaitis*, et al*., 2021). The mean and standard error of the mean were used to represent descriptive statistical variables.

RESULTS AND DISCUSSIONS

Bovine mastitis initially affects only one mammary quarter, after which the infection may also spread to the others. For this reason, the diagnostic tests were performed individually (individual approach) and for each mammary quarter separately (topic-glandular approach). Figure 1 depicts the several steps employed in the diagnostic of bovine mastitis, as well as the various therapeutic choices based on the diagnostic findings.

The presence of inflammatory changes in the mammary gland may be characterized by an increase in the number of somatic cells. Previous studies have shown that permeability changes related to inflammatory reactions in secretory cells can cause changes in milk components, such as the percentage of protein, fat and lactose, as well as a decrease in milk production (Table 1).

Early detection of bovine mastitis may help improve treatment strategies, increase milk production and reduce antibiotic use on farms. Thus, current scientific research focus is being orientated in identifying new and less expensive biomarkers for early diagnosis. One such proposed biomarker is the percentage of lactose in milk.

In our study, we investigated the possible correlations between the somatic cell score (SCS) and other physico-chemical parameters of milk, in healthy cows and in cows with mastitis, using the Pearson correlation coefficient and linear regression. A very small significant negative relationship was observed between somatic cell score and lactose percentage, ($r = 0.72$, $p < .001$) (Figure 2).

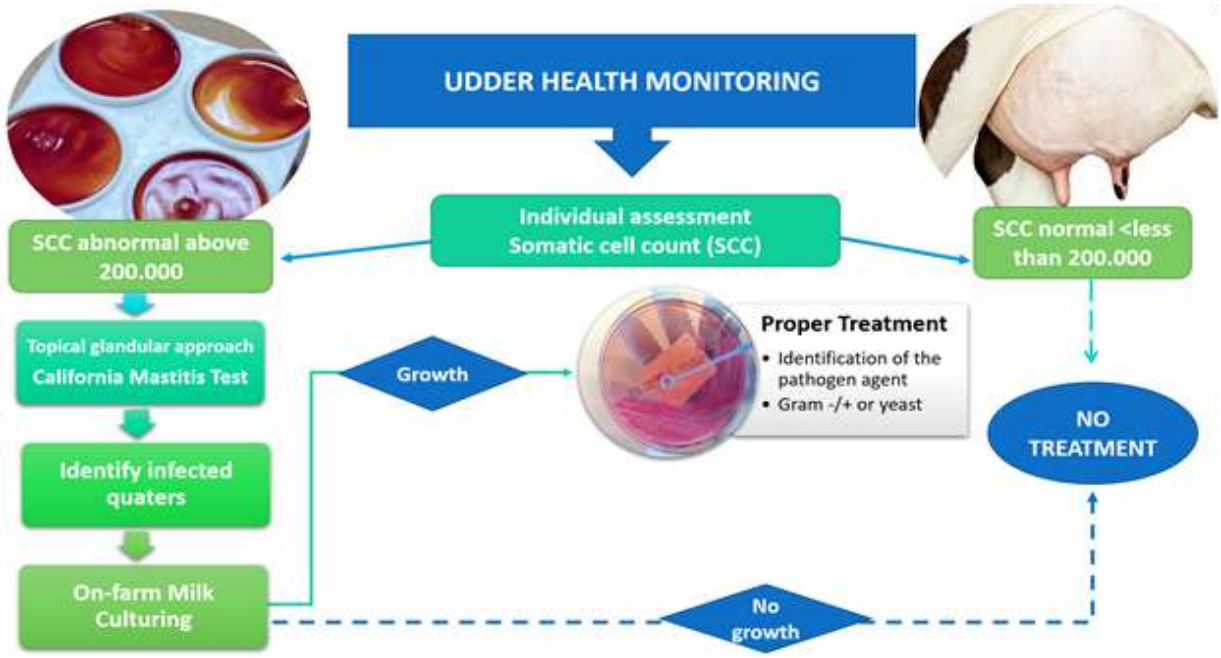

Figure 1 **Diagram of decision making for diagnosing and treatment of mastitis on dairy farms**

Table 1

Descriptive statistical variables of the physico-chemical parameters of milk in cows with mastitis and healthy cows

Type	Mastitis (n=19)	Healthy (n=50)	*p-value*
SOMATIC CELL SCORE	15.10 ± 2.25[a]	12.36 ± 1.19	< 0.0001
FAT (%)	3.48 ± 0.82[b]	3.96±0.89	0.0488
PROTEINS (%)	3.39±0.51[c]	3.10±0.27	0.0033
LACTOSE (%)	4.65±0.68[d]	5.04±0.22	< 0.0001
DRY MATTER	12.52±1.78[e]	11.65±1.02	0.0063
CASEIN (%)	2.54±0.23[f]	2.79±0.42	0.0028
a,b,c,d,e,f - Superscript letters indicate that the difference is considered statistically significant.			

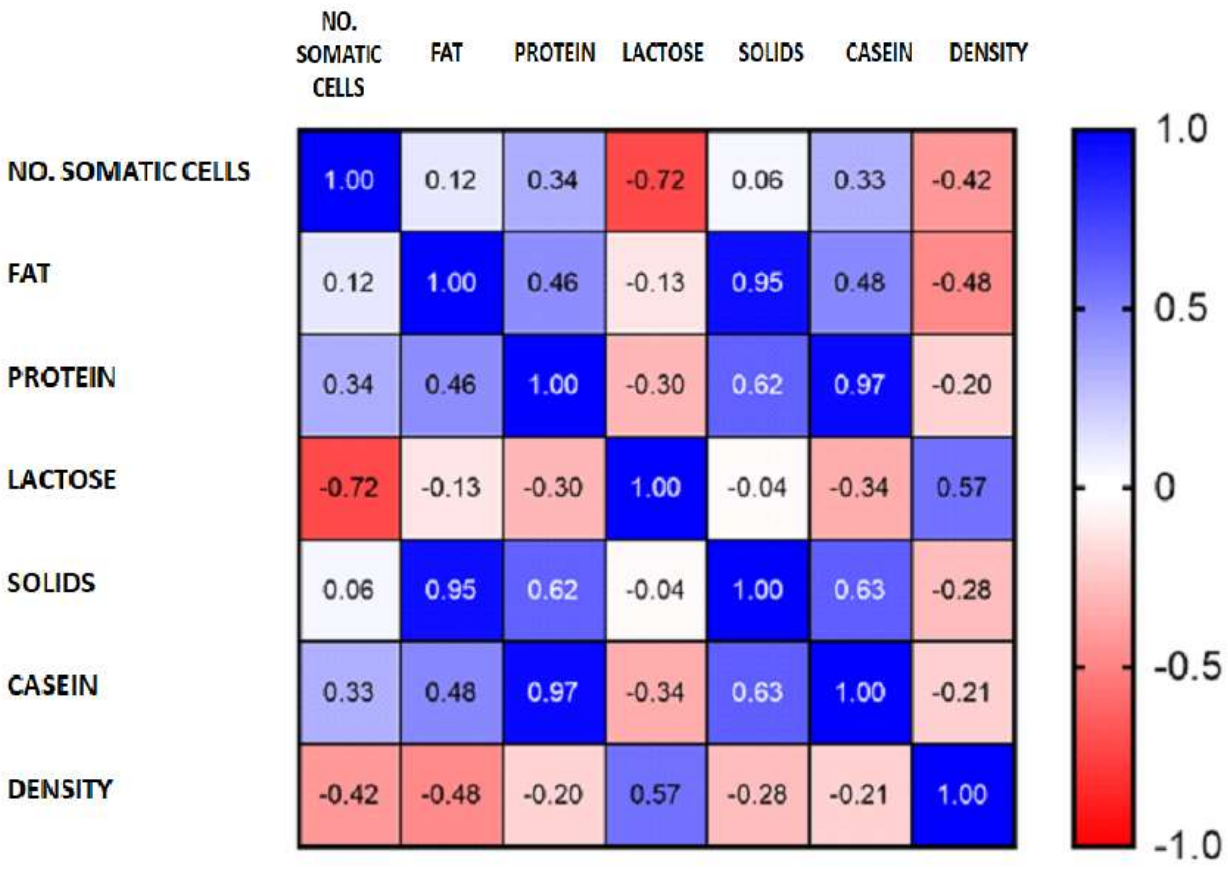

Figure 2 **Correlations between the physico-chemical parameters of milk and the somatic cell score (SCC)**

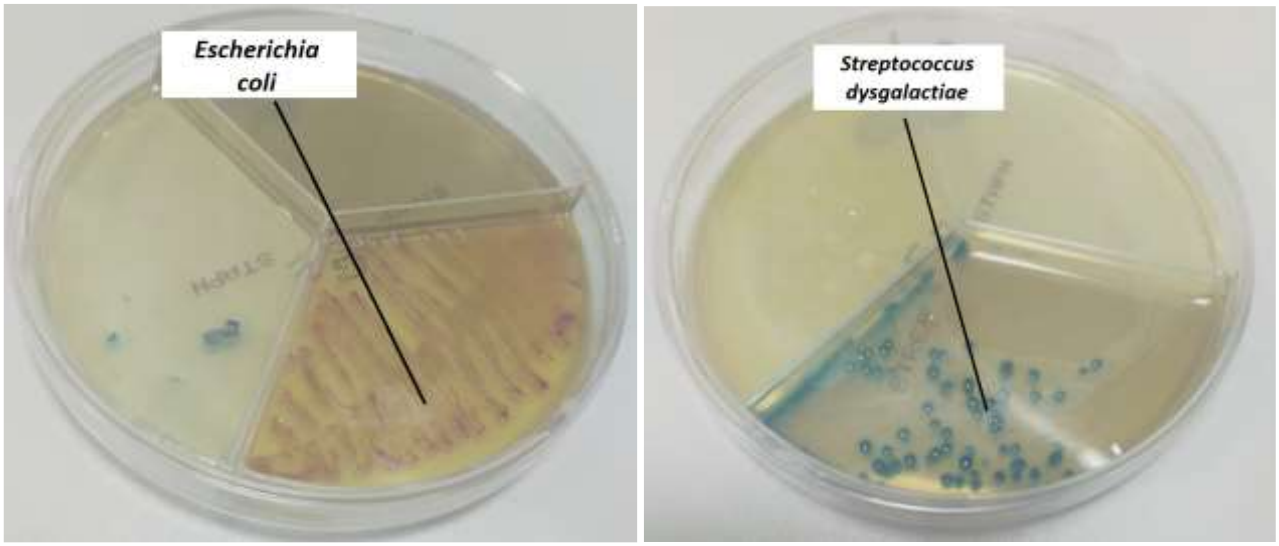

Figure 3. **Pathogen identification in milk samples from cows with clinical mastitis using the Clear Milk test**

Identifying the pathogen agent responsible for inducing bovine mastitis, samples collected from cows with visible signs of clinical mastitis, as well as milk samples with a somatic cell count above the threshold of 200.000 cells/ml were analyzed using the Clearmilk kit (Figure 3).

The on-farm culturing system allows practitioners to distinguish between pathogens. Antibiotic treatment may be chosen for mastitis cases that are likely to benefit from this type of therapy, such is the case of mastitis generated by Gram-positive pathogens (*Strep. dysgalactiae*, *Strep. uberis*, or coagulase-negative staph.). In the case of Gram-negative infections (E. coli or Klebsiella) antibiotics should not be used unless it becomes toxic (Smulski et al., 2020).

In our study, the most frequent encountered pathogen agents were *Streptococcus spp*. A narrow group of bacteria (*Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae*, and *Escherichia coli*) are accountable for 80% of mastitis cases, with *S. uberis* consistently identified as the predominant pathogen in dairy farms worldwide (Maciel-Guerra *et al.*, 2021).

Reducing udder pathogen exposure is one of the most effective mastitis prevention methods and may be achieved by implementing proper milking parlor procedures, such as adequate pre-dipping, ensuring that the whole teat skin is coated to the base while providing adequate time for the pre-dipping solution to kill the pathogens, and subsequently dry wiping of teats (Ruegg, 2017).

CONCLUSIONS

A management approach for mastitis, as well as other diseases, should include the following major components: prevention, monitoring, and treatment. Due to the fact that mastitis is a pluri-factorial disease, the preventative component should target the primary risk factors for mastitis. Independently of the kind of mastitis and the pathogen agent, there are three basic farm management aims that should be considered: stimulation of the udder's natural defensive systems, ensuring complete and proper milking and lowering udder pathogen susceptibility.

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INDUCING OVULATION WITH HUMAN CHORIONIC GONADOTROPHIN IMPROVES CUMULATIVE PREGNANCY RATES OF DAIRY COWS DURING THE WARM SEASON

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Abstract
This study was designed to assess the effects of the GnRH agonist gonadorelin and human chorionic gonadotrophin, during a timed artificial insemination program, on the fertility of lactating dairy cows during the warm and cold seasons of the year. Cows were treated intramuscularly with GnRH-agonist (Day 0) and PGF2α (Day 7), followed by either GnRH-agonist (GPG treatment; 38 animals) or hCG (GPH treatment; 26 animals) on Day 9. All cows were fixed-time inseminated (TAI) 16–22 h after the end of treatment. In this study, replacing the Day 9 dose of GnRH with a dose of hCG may result in an increase in the cumulative pregnancy rate (46.6% vs. 25%, $P \leq 0.05$) but not the pregnancy rate (26.6% vs. 18.8%, $P > 0.05$) in heat-stressed cows (THI > 70). However, in cold season, no effects were observed in the pregnancy rates or cumulative pregnancy rates of the examined cows. This finding indicates that hCG therapy in TAI programs may mitigate the negative effects of heat stress on dairy cows reproduction. This tendency can be explained also by some studies that have shown that hCG can significantly reduce the number of small luteal cells while increasing the number of large luteal cells as the corpus luteum gets bigger in diameter, area, and volume.

Key words: hCG, TAI, pregnancy rate, heat stress, dairy cows.

INTRODUCTION

The dairy sector has created and extensively implemented timed artificial insemination (TAI) programs to get over the limitations of estrus detection and enable scheduled artificial insemination (Caraviello et al., 2006; Pursley et al., 1995). However, between 40 and 60 percent of the treated animals do not conceive at the time of TAI. The failure of TAI could be caused by a certain number of factors.

For exemple, 12–21% of cows given a single dose of PGF showed incomplete luteal regression in a Ovsynch protocol (Wiltbank et al., 2015; Brusveen et al., 2009; Carvalho et al., 2015). A single dose of prostaglandin F2α (PGF) cause incomplete luteal regression which is the source of increased progesterone (P4) concentration at the second GnRH treatment.

In cows that began a TAI program in a low P4 condition, the subsequent decline in pregnancy per AI (P/AI) may be significant (Giordano et al., 2012; Carvalho et al., 2015; Santos et al., 2016). In addition, increasing P4 at the beginning of the treatment has a favorable influence on the growth of the dominant follicle and luteal regression, hence improving fertility (Bisinotto et al., 2011; Pursley et al., 2011; Borchardt et al., 2018). Another factor which cause failure of TAI protocol is low circulating P4 after breeding which may impact embryo development and maternal recognition of pregnancy (Mann and Lamming, 2001). Large luteal cells are more widespread after human chorionic gonadotrophin (hCG) therapy in cows (Helmer and Britt, 1987; Sianangama and Rajamahendran, 1992) which is also associated with a decrease in smaller luteal cells and an increase in plasma P4 levels (Breuel et al., 1989; Fricke et al., 1993; Diaz et al., 1998). In some studies, hCG is administered as part of the Ovsynch protocol to synchronize and induce ovulation (De Rensis et al., 2002; Schmitt et al., 1996) in order to improve fertility. According to Kinser et al. (1983), in contrast to GnRH, hCG has a longer half-life than natural LH and works independently of the pituitary gland.

Thus, the aim of the present study was to evaluate the effects of hCG administration within an Ovsynch protocol on the fertility in lactating dairy cows during the warm and cold seasons.

MATERIAL AND METHOD

This study was focused on a Holstein-Friesian dairy herd located in the North-Eastern part of Romania. During the studied period, the average number of lactating cows in the herd was approximately 340, and the average annual milk production per cow was about 8,000 kg milk/305 days. According on their body condition score and whether or not they were diagnosed as being pregnant with twins, dry cows were housed in a separate group and transferred 21 days prior to birth to the "parturition group" (close-up group). The cows were housed in free-stall barns with concrete floors and a straw bed, their feeding being based on a Total Mixed Ration twice daily with *ad libitum* access to water, according on their milk production and cow size. In order to maintain the animals health, standard management procedures were used.

In order to reduce variation, only animals that corresponded in terms of a good health and physical condition at the beginning of treatments were chosen. Thus, only cows scoring between 2.5 and 3.5, which were regarded to be in a good body condition, were included in the trial (Gearart et al., 1990). Regarding the voluntary waiting period, a minimum of 60 days after parturition have been set.

Estrus cows were detected from the AfiMilk (AfiMilk, Kibbutz Afikim, Israel) estrus daily report and each animal was examined by an experienced veterinarian. The indications of estrus signs were represented by the desire to mount other cows, chasing herd members, restlessness, chin resting, sniffing herd mates vagina and bellowing, congestion, relaxation, and mucus secretion from the vulva. The behavior of standing to be mounted was regarded as an indication of real estrus signs.

Two groups of milking cows were organized to conduct this study, as following: **GPG group** (38 dairy cows): (Day 0) GnRH (Gonavet Veyx, Veyx-Pharma GmbH, Germany, 100 micrograms of Gonadorelin[6-D-Phe], i.m.) followed by PGF (PGF Veyx Forte, Veyx-Pharma GmbH, Germany, 0.5 mg cloprostenol/cow, i.m.) 7 days later and then a second GnRH dose 36 h after the PGF and AI after 16h. The second group was presented by **GPH group** (26 animals): (Day 0) GnRH (Gonavet Veyx, Veyx-Pharma GmbH, Germany, 100 micrograms of Gonadorelin[6-D-Phe]/cow, i.m.) followed by PGF (PGF Veyx Forte, Veyx-Pharma GmbH, Germany, 0.5 mg cloprostenol/cow, i.m.) 7 days later, and then hCG (1500 IU, Chorulon, MSD, Holland) given 36 h after the PGF and AI after 16h.

For all the animals included in the study, AI was performed by two operators with over 20 years of experience. The pregnancy diagnosis was performed with the Draminski iScan2 veterinary ultrasound instrument (Draminski S.A., Poland).

Statistical analysis

Depending on the temperature humidity index (THI), we established two distinct climate periods in the studied area: warm (May to September, THI exceed 70) and cold (October to April, THI below 70). Thus, all the data regarding the performed treatments were used to compare the effect of treatments on reproductive success during warm and cold periods. To determine the pregnancy rate at TAI, we evaluated the percentage of animals among the total number of cows in the relevant group that became pregnant after receiving TAI. The cumulative pregnancy rate was calculated as the proportion of cows in each group who got pregnant after two sessions of artificial insemination (first AI plus return AI) within 30 days of the completion of treatment.

The comparative statistical analysis between GPG and GPH groups in terms of pregnancy rate and cumulative pregnancy rate was performed by applying the Chi-square test (Thrusfield, 1995). The level of statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

Table 1 highlights the reproductive performance of dairy cows subjected to the GPG and GPH protocols during the heat stress period compared to non-heat stress periods of the year. It was observed that in TAI programs, the pregnancy rates for the two groups of cows in heat stress periods did not show significant differences compared to periods with THI < 70. However, a significant difference between the two protocols was observed ($P < 0.05$), in terms of the cumulative pregnancy rate during the heat stress period (THI > 70).

Regarding TAI programs, replacing the day 9 dose of GnRH with a dose of hCG may result in an increase in the cumulative pregnancy rate in stressed heat cows (THI > 70). This indicates that hCG treatment can counteract the detrimental effects of heat stress on the reproduction of dairy cows. This can be explained by the fact that, according to some studies, hCG has the ability to cause a significant decrease in the number of small luteal cells (Farin et al., 1988; Helmer et al., 1992), while the number of large luteal cells increases proportionally with the corpus luteum's diameter, area, and volume (Rajamahendran and Sianangama, 1992; Santos et al., 2001). All of these actions serve to promote dairy cow fertility by boosting luteal activity and, consequently, serum progesterone levels (De Rensis et al., 2008). In a study conducted by De Rensis et al. (2002), it was observed that the hCG product administered in OvSynch programs is less successful than the GnRH product. This can be explained by the fact

that the animals included in our study, as in the study carried out by De Rensis et al., (2008), were selected to be part of the two groups only if by ultrasound examination, the presence of a large follicle and a corpus luteum in the structure of the ovaries at the beginning (day 0) of the GPG and GPH protocols were detected.

Table 1

Pregnancy rate at TAI and cumulative pregnancy rate in dairy cows after applying the protocol GPG (GnRH on day 0, PGF2α on day 7, GnRH on day 9 and AI at 16h) and GPH (GnRH on day 0, PGF2α on day 7, hCG on day 9 and AI at 16h)

Reproductive parameters	THI	Group GPG	Group GPH	Statistical significance
Pregnancy rate at TAI	< 75	50% (11/22)	54.5% (6/11)	P > 0.05
	> 75	18,8% (3/16)	26.6% (4/15)	P > 0.05
Cumulative pregnancy rate	< 75	72.7% (16/22)	81.8% (9/11)	P > 0.05
	> 75	25% (4/16)	46,6% (7/15)	P ≤ 0.05

CONCLUSIONS

The cumulative pregnancy rate during the heat stress period is significantly increased when hCG is used in TAI programs, assuming that females undergoing these protocols have developed follicles and the corpus luteum at the time when protocol was initiated.

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IMPROVING FERTILITY IN MULTIPAROUS COWS BY INDUCING OVULATION WITH GNRH AGONIST - GONADORELIN OR HUMAN CHORINIC GONADOTROPHIN

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Abstract
This study was designed to assess the effects of the GnRH agonist gonadorelin and human chorionic gonadotrophin administered at first artificial insemination on the fertility of lactating dairy cows. The lactating dairy cows were divided into three groups: the hCG-group consisting of primiparous and multiparous cows that expressed the specific behavior of estrus with AI 12 hours after the end of estrus along with administration of 1500 IU of human chorionic gonadotropin (hCG); the GnRH-group consisting of primiparous and multiparous cows that showed the specific behavior of estrus with AI 12 hours after the end of estrus along with administration of 2 mL of Gonadorelin; and the control group (C-group) consisting of primiparous and multiparous cows that showed the behavior specific to estrus and that were artificially inseminated (AI) 12 hours after the end of estrus signs without any treatment. According to our results, administering GnRH agonist or hCG products to multiparous cows in order to induce ovulation increases the pregnancy rate during the first artificial insemination (46.6%, 57.1% vs. 22.2%, $P \leq 0.05$). Both products don't have any effects on primiparous dairy cows (45%, 42.9% vs. 50%, $P > 0.05$).

Key words: GnRH agonist, hCG, dairy cows, ovulation, multiparous.

INTRODUCTION

More commonly, producers face the issue of infertility in dairy cows. In spite of numerous research on the topic, the reproductive efficiency of dairy cows has recently declined. This downward tendency is primarily due to the low rate of oestrus identification and ovulation timing. In the past, it was assumed that the low rate of oestrus detection was due to the farmer's lack of interest because of other farm priorities (Alexander et al., 1984). As research on this area has developed, it has become clear that a number of farm-level parameters have a significant effect on the rate of estrus detection and, implicitly, the timing of ovulation (Dransfield et al., 1998; Stevenson et al., 1998; Van Vliet and Van Eerdenburg, 1996).

Artificial insemination (AI) is regarded as the most effective approach for achieving high pregnancy rates in dairy cows, and the behavior of other cows accepting to be mounted is considered to be a specific indicator for determining the optimal time to do AI. (Bostedt, 1976; Maatje et al., 1997; Nebel et al., 2000; Trimberger, 1948; Walker et al., 1996).

Very few studies have examined the effect of hormone therapy for ovulation management in dairy cows on reproductive performance. According to Chenault et al. (1990), a single dose of the GnRH analog buserelin induces the release of LH at the pituitary level for about 5 hours, which is approximately half the duration of the hormone's normal release from the pituitary level (Rahe et al., 1980). This phenomenon can interfere with both ovulation and the subsequent development of the corpus luteum.

Human chorionic gonadotropin is an additional hormone that may be used in ovulation induction methods (hCG). It appears that hCG-based products are more effective than GnRH-based treatments because they indirectly improve luteal function, embryo viability, and fertility (Schmitt et al., 1996, Mee et al., 1990; Stevenson et al., 1990; Khan et al., 2003). However, it is widely acknowledged that hCG and GnRH products have similar effects on ovarian function (Rajamahendran and Sianangama, 1992; Fricke et al., 1993), with the specification that hCG operates independently on the pituitary gland and has a longer duration of action than spontaneous LH discharges (Kinser et al., 1983).

Thus, the objective of this study was to examine the ovarian structures using ultrasound during dairy cows' estrous behavior in connection to parity-based pregnancy rates. In addition, the influence of GnRH and hCG-induced ovulation on pregnancy rates was assessed.

MATERIAL AND METHOD

Northeastern Romanian Holstein-Friesian dairy herd served as the subject of this study. The average number of lactating cows in the herd was 340 during the study period, and the average annual milk output per cow was around 8000 kg milk/305 days. Depending on their body condition score and whether or not they were pregnant with twins, dry cows were kept in a separate group and moved to a "parturition group" 21 days before the parturition (close-up group). The cows were housed in free-stall barns with concrete floors, straw bed and fed a Total Mixed Ration twice per day with *ad libitum* water access, according to the level of milk production and cow size. To keep the animals healthy, standard management practices, were used.

All ovarian follicles with a diameter of less than 5 mm were measured by transrectal ultrasonography examination (Draminski iScan2 veterinary ultrasound, Draminski S.A., Poland) to evaluate the size of the mature ovarian follicle, close to ovulation. The follicle with a diameter higher than 10 mm and revealing at least 2 mm more than the other follicles was referred to as the dominant (mature or preovulatory) follicle (Sirois and Fortune, 1990).

A minimum of 60 days following calving have been designated as the voluntary waiting period.

The AfiMilk (AfiMilk, Kibbutz Afikim, Israel) estrus daily report was used to identify cows in estrus, and each one was examined by an experienced veterinarian. The indications of estrus included attempts to mount other cows, chasing herd mates, restlessness, chin resting, sniffing herd mates' vagina and bellowing, congestion, relaxation, and mucus discharge from the vulva. The presence of standing estrus was considered to be a sign of true estrus.

Three groups of milking cows were organized to conduct this study: control group (group M) consisting of primiparous and multiparous cows that showed the specific behavior of estrus and that were artificially inseminated (IA) 12 hours after the end of estrus; GnRH group consisting of primiparous and multiparous cows that showed the specific behavior of estrus, AI 12 hours after the end of estrus, with the administration of 2 ml of Gonadorelin (Gonavet, VEYX PHARMA GmbH, Germany) and the hCG group consisting of primiparous and multiparous cows that showed the specific behavior of estrus, AI 12 hours after the end of estrus signs with the administration of 1500 IU hCG human chorionic gonadotropin (MSD, Animal Health, USA). For all cows included in the study, AI was performed by two operators with over 20 years of experience. The pregnancy diagnosis was performed using the Draminski iScan2 veterinary ultrasound (Draminski S.A., Poland).

Statistical analysis

The comparative statistical analysis between control and experimental groups in terms of conception and pregnancy rates was performed by applying the Chi-square test (Thrusfield, 1995). The comparative evaluation (primiparous vs. multiparous) of the intervals until the first detected estrus, of the voluntary waiting periods, the Interval between artificial inseminations (postpartum days, mean ± SD) was performed by applying Mann's U test-Whitney. The level of statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSIONS

Table 1 displays the reproductive parameters associated with the ovarian activity of the dairy cows on the studied farm. Multiparous cows have a longer interval between their first detectable estrus compared to primiparous cows (P 0.05). We can therefore hypothesize that uterine involution in multiparous cows requires a longer postpartum period than in primiparous cows. However, this aspect had no effect on the postpartum interval to the administration of the first dosage of semen in primiparous cows compared multiparous cows (Table 1, $P > 0.05$).

After performing the pregnancy diagnosis, it was observed that the conception rate of primiparous cows did not differ significantly from that of multiparous cows, with values of 47.5% for primiparous cows and 39% for multiparous cows.

In order to continue evaluating reproductive activity, the interval between estrus was determined in non-pregnant cows that received a second or third dose of semen to increase the proportion of pregnant cows on the farm. Therefore, in this study, the interval between estrus signs tended to be longer for multiparous cows than for primiparous cows (Table 1).

In a study conducted by Remnant et al. (2014), the interval between estrus was analyzed, the results revealing that this parameter has a tendency to increase with parity.

Withal, Diskin et al. (2011) observed that increased values of the intervals between the first and second AI and between the second and third AI were associated with the presence of early embryonic death. Current studies on this topic indicate that early embryonic death is higher in multiparous cows compared to primiparous cows (El-Tarabany et al., 2016). The explanation for this is given by Kafi et al. (2015), who found that dairy cows achieve excessive body condition score losses, and this fact causes a disruption of the length of the luteal phase and implicitly of

maintaining the functionality of the corpus luteum after calving.

The results of this study are presented in table 2. Thus, in this study, in primiparous cows, the pregnancy rate at the first artificial insemination does not show differences ($P > 0.05$) between the three groups of cows.

However, in the case of multiparous cows, the pregnancy rate at the first AI was significantly higher ($P \leq 0.05$) in the GnRH and hCG groups compared to the M group. In addition, in group M, it was observed that there was a difference (50% vs. 22.2%, $P = 0.001$) in the first AI pregnancy rate between primiparous cows compared to multiparous cows (Table 2).

CONCLUSIONS

In conclusion, the administration of hormonal products based on GnRH and hCG at the time of AI can improve the pregnancy rates at the first AI in multiparous cows.

Table 1

Reproductive indicators of dairy cattle in selected dairy farms

Reproductive parameters	Primiparous (n = 158)	Multiparous (n = 182)	Total (n = 340)
First detected estrus (days postpartum, M ± SD)	50 ± 19.2[b]	71 ± 14.3[a]	66 ± 19,1
Adjusted voluntary waiting period (days postpartum, M ± SD)	77.75 ± 42.23	82.29 ± 37.19	79.95 ± 39.84
Conception rate (%)	47.5 %	39 %	42.9 %
AI1 – AI2 interval (days postpartum, M ± SD)	38.61 ± 23.28	45.67 ± 27.38	42.3 ± 25.66
AI2 – AI3 interval (days postpartum, M ± SD)	34.11 ± 19.65	40 ± 21.3	36.78 ± 20.52

AI1 - first artificial insemination; AI2 - second artificial insemination; AI3 - third arificial insemination; M ± SD - mean ± standard deviation. [a,b] Values with different superscripts within rows denote significant differences between groups detected by the Mann-Whitney test (a vs. b, a > b, P < 0,05), M ± SD = mean ± standard deviation.

Table 2

Ovulatory response of dairy cows in experimental groups subjected to treatment with (GnRH, hCG) compared to group M (no treatment). Cows in the experimental groups were given single doses of GnRH and hCG at the time of AI

Items	Group M		Group GnRH		Group hCG	
	Primiparous (n = 55)	Multiparous (n = 73)	Primiparous (n = 60)	Multiparous (n = 43)	Primiparous (n = 34)	Multiparous (n = 33)
Ovulatory follicle diameter (mm, M ± SD)	16.04 ± 5.39	15.29 ± 4,33	16.46 ± 4.03	17.55 ± 4.2	17.27 ± 5.69	15.69 ± 5
Pregnancy rate at first AI (%)	50[d] (9/18)	22.2[b,c] (6/27)	45 (9/20)	46.6[a] (7/15)	42.9 (6/14)	57.1[a] (8/14)
Pregnancy rate at two and more artificial inseminations(%)	35.1 (13/37)	32.6 (15/46)	42.5 (17/40)	33.3 (9/27)	40 (8/20)	31.6 (6/19)

[a,b] Values with different superscripts within rows denote significant differences between groups detected by the chi-square test (a vs. b, a > b, P<0.05); [c,d] Values with different superscripts between primiparous and multiparous cows; M ± SD = mean ± standard deviation.

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A CASE OF PERICARDITIS IN ANGUS CATTLE

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Abstract

A 10-years-old Angus cow was evaluated on the field for suspected traumatic pericarditis. Physical exam findings included tachycardia, mixed dyspnea, decreased intensity of heart sounds on the left, absence of heart sounds on the right but presence of fluid sounds, bilateral turgidity of the jugular veins, positive venous compression test, brisket and ventral abdominal oedema. The cow exhibited light pain while palpation at brisket region. Hematology revealed significant erythrocytopenia, higher hematocrit, lower hemoglobin concentration, significant leukocytosis with neutrophilia and lymphocytopenia. Serum biochemical findings had reduced glucose, urea nitrogen, albumin, sodium, potassium, chloride, calcium, phosphorus levels and increased levels of AST, ALT, GGT, total protein and globulin. Ultrasonography has shown pericardial effusion and strands of fibrin. The prognosis was poor, and pericardiocentesis or pericardiotomy were inadequate methods of treatment, thus euthanasia was indicated.

Key words: traumatic pericarditis, bovine, ultrasound

Pericarditis is an inflammation of the pericardium that results in the accumulation of serous or fibrinous inflammatory products between the visceral and parietal pericardium. It can be caused by trauma from ingested foreign objects (nails, needles, wire), external injuries, hematogenous spread of infectious diseases (colibacillosis, pasteurellosis, salmonellosis and anaerobic infections) (Grunder H.D., 2002), neoplastic effusion secondary to a lymphoma or a mesothelioma (Takasu M. et al., 2006), and idiopathic aseptic pericarditis (Jesty S.A. et al., 2005; Firshman A.M. et al., 2006).

In cows, pericarditis is almost always attributable to a reticular foreign body that has penetrate the reticular wall, diaphragm and pericardial sac (Braun U., 2009). The primary clinical sign in susceptible cattle is tachycardia and the severity of this sign depends primarily on the degree of compression of the heart by pericardial effusion (Jesty S.A. et al., 2005). However, tachycardia may also be secondary to various disorders such as dehydration, anemia, hypoxemia, acid–base and metabolic disturbance, hypovolemia, pain, fever, excitement, stress, sepsis or toxemia.

This case report highlights the value of clinical exams and echocardiography for an antemortem diagnosis of pericarditis in cows.

MATERIAL AND METHOD

A ten-years-old Angus cow was evaluated on the field for suspected traumatic pericarditis. The cow had been maintained on pasture with other 10 healthy cattle and had been apparently healthy until one month after calving, when the owner first noticed a sharply decreased milk production and diminished appetite. The veterinarian administered benzyl procaine penicillin and dexamethasone for 3 days. Its evolution was favorable, milk production increased a little but remained below normal for cows in their second month of lactation. Two months after calving, the owner noticed that the cow's brisket area was getting bigger and her milk production had dropped to 3-4 liters. The owner has contacted the specialists from Iasi University of Life Sciences. A complete clinical examination was performed. Whole blood and serum samples were collected to determine various hematological parameters and serum concentrations of aspartate aminotransferase (AST), urea nitrogen, gamma glutamyl transferase (GGT), total protein, alanine aminotransferase (ALT), albumin, glucose, sodium, potassium, chloride, calcium and phosphorus. Ultrasonographic examination of the heart has been performed on standing cow using a 5.0 MHz convex transducer from the third to fifth

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intercostal spaces in the cardiac region on both sides of the thorax.

RESULTS AND DISCUSSIONS

Heart disease in cattle is a challenge for the clinician. The bovine heart is composed of three major structures that can be affected by various diseases: the pericardium, the myocardium and the endocardium. Various congenital anomalies of the heart occur in cattle as the result of defective septa and formation of the cardiac chambers during embryonic development (Buczinski et al., 2005). The most common congenital heart defect is the incomplete closure of the ventricular septum (Ohwada and Murakami, 2000; Buczinski et al., 2006). The most prevalent endocardial disease is infectious bacterial endocarditis mainly caused by *Trueperella pyogenes* or streptococci. The most prevalent disease of the myocardium is dilated cardiomyopathy which is a primary myocardial disorder (Nart et al., 2004). Secondary myocardial disorders include myocarditis following viral (Gunes et al., 2005), bacterial (Uzal et al., 2003; Haines et al., 2004) or parasitic infection, nutritional cardiomyopathy such as that caused by vitamin E/selenium or copper deficiency, and toxic cardiomyopathy secondary to ionophore toxicosis. Pericardial diseases include traumatic reticulopericarditis, pericarditis secondary to pleural or lung infection, neoplastic effusion secondary to a lymphoma or a mesothelioma, and idiopathic aseptic pericarditis (Buczinski S. et al., 2010).

The prognosis is traditionally said to be guarded to poor in bovine heart disease cases. However, diagnostic tools are available to detect cardiac disease before signs of heart failure appear. Auscultation is the most important part of the examination of the cardiovascular system in cattle, as most are examined in the field without access to additional diagnostic tools. This procedure should be carried out systematically with the aim of detecting normal heart sounds, cardiac murmurs and cardiac rhythm.

The Angus cow had as clinical signs tachycardia (100 bpm), normothermia, mixed dyspnea, decreased intensity of heart sounds on the left, absence of heart sounds on the right but presence of fluid sounds, bilateral turgidity of the jugular veins, positive venous compression test, brisket and ventral abdominal oedema (Figure 1 a,b,c), reduced rumen movement and positive pain tests.

The primary clinical sign in affected cattle is tachycardia. Sometimes the heart rate is only mildly elevated at 80–100 bpm; typically, however, the heart rate is severely increased with rates as high as 130 bpm (Braun U., 2009). The severity of tachycardia depends mainly on the degree of compression of the heart by the pericardial effusion; in cows with idiopathic pericarditis, pericardiocentesis with fluid removal resulted in an immediate decrease in blood pressure, heart rate and recovery (Jesty S.A. et al., 2005).

Heart sounds are suppressed due to pericardial effusion and fibrinous changes in the pericardial sac. These abnormal sounds depend on the type of lesions (Grunder H.D., 2002). If fibrinous changes, the sound are of rubbing and scratching nature. With a predominance of fluid, there are splashing or gurgling sounds.

There is a variable degree of jugular vein distention, depending on the degree of cardiac tamponade (Jesty S.A. et al., 2005; Braun U. et al., 2007), as well as edema of the submandibular region, chest and ventral abdomen. Sometimes cattle stay with their elbows in abduction in an attempt to facilitate cardiac function. Elbow abduction can also be the result of pain. It is possible that oedema and jugular vein distention may not be present if pericardial fluid drains into the reticulum via a patent foreign body tract (Grunder H.D., 2002). Clinical signs of heart failure in cattle include syncope, exercise intolerance and weakness (De Morais and Schwartz, 2005). Congestive heart failure can be distinguished from heart failure by the presence of effusion and oedema, which are the result of increased hydrostatic pressure and fluid retention (Reef and McGuirk, 2002).

The general behavior, condition and appetite of cattle with traumatic pericarditis are always abnormal. Cattle often show signs of pain, such as bruxism and grunting, and fever. Absence of fever does not exclude traumatic pericarditis. Respiratory rate is often elevated due to heart failure or direct lung involvement, and ruminal motility is usually reduced or absent. Because pericarditis typically results from traumatic reticuloperitonitis, tests for reticular foreign bodies are positive in 85% of affected cows (Braun U. et al., 2007).

The blood findings in cases of heart disease are non-specific. In Angus cow hematological abnormalities were leukocytosis (17.31 x 10^9/L, normal 4.9–12) with neutrophilia (9.42 x 10^9/L, normal 1.8–6.3), lymphocytopenia (0.41 x 10^9/L, normal 1.6–5.6) and normocytic hypochromic anemia (RBCs 3.50 x 10^{12}/L, normal 5.1–7.6 x 10^{12}/L; Hgb 6.5 g/dL, normal 8.5–12.2; HCT 35.9 %, normal 22–33). Albumin (1.8 g/dL, normal 3.03–3.55) and urea nitrogen (15 mg/dL, normal

20–30) from serum had values below the lower physiological limit, indicating hepatic insufficiency. Parameters such as ALT (89 IU/L, normal 11–40), AST (189 IU/L, normal 78–132), and GGT (175 IU/L, normal 15–39) were found in the serum in elevated levels, which are associated with possible hepatocellular damage and cholestasis. The total bilirubin level (0.8 mg/dL, normal 0.01–0.5) was increased associated with hepatocyte damage, cholestasis, and hemolytic anemia. The activities of GGT, AST and the serum concentration of bilirubin are increased, indicating hepatic congestion. An increase in the activities of liver enzymes, especially GGT, in cattle with right-sided cardiac insufficiency is usually a sign of liver congestion and not primary liver disease (Braun U., 2009). Serum globulins (8.1 g/dL, normal 3–3.48) and total protein (10 g/dL, normal 6.74–7.46) had values that exceeded the upper physiological limit, indicating infection. The serum activities of glucose, phosphorus, calcium, sodium, and potassium were slightly decreased according to the parameters for the species (Kaneko, J.J. et al., 2008). Inflammatory processes and different degrees of renal, hepatic, and muscular damage can be detected. A case report of idiopathic pericarditis demonstrated a correlation between serum cardiac troponin I (cTnI) levels and the severity of the disease. A decrease in cTnI levels was also correlated with improvement of the cow (Jesty et al., 2005).

Figure 1 a,b,c. Oedema of the abdominal region and brisket and distension of the jugular veins in an Angus cow with traumatic reticuloperitonitis and pericarditis

A sample of pericardial fluid may be aspirated from the pericardial sac. The smell, reminiscent of retained placenta and toxic metritis, is sufficiently diagnostic in cattle with traumatic pericarditis (Radostits et al., 2007). The technique is not without danger, however, as infection may spread to the pleural cavity.

Traumatic pericarditis can be diagnosed on radiography when a foreign body is seen perforating the cranial reticular wall and diaphragm, or is situated entirely cranial to the reticular wall. For an accurate location of a foreign body, a dorsoventral radiographic view would be necessary, but this is not possible in adult cattle because of the dorsoventral depth of the thorax (Braun U., 2009). Traumatic pericarditis cannot however be ruled out if a foreign body is not seen as thick radiodense adhesions may obscure a foreign body from view or the foreign body may have migrated back into the reticulum, where it may be seen lying freely in the organ or penetrating the reticular wall (Braun U., 2009). Thoracic radiographs are more helpful in small animals with heart disease to assess heart and vessel sizes and pulmonary oedema (Buchanan, 2000). Recently, radiographic measurement of the caudal vena cava has been mentioned as a reliable tool for detecting heart disease in cattle because in cases of heart disease, the caudal vena cava had a decreased or absent pulsation index because of its severe distension (Jilintai et al., 2006).

The diffuse organisation of the Purkinje fibres in the bovine myocardium does not facilitate good ECG sensitivity (Reef and McGuirk, 2002). Consequently, the ECG cannot be used to detect enlargement of the cardiac chambers in cattle, although it is useful for differentiating physiological from pathological arrhythmias.

Ultrasonography is the method of choice for imaging and characterizing pericardial effusion. Cardiac ultrasonography has been described as a reliable tool to detect various types of heart disease. However, at this time, the usefulness of this non-invasive imaging process is limited to the diagnosis of heart disease and has no proven prognostic capabilities. Unlike in small animals or horses (Reef, 1995), echocardiographic measurements of ventricular septal defect sizes had no prognostic value in cattle (Buczinski et al., 2006). Prospective studies with the objective of finding prognostic parameters therefore still need to be performed in the bovine species. The

presence of large quantities of fibrin in the pericardium has been suggested as an important prognostic parameter in cases of pericarditis.

The Angus cow had a large amount of hypoechogenic pericardial fluid, and echogenic deposits and strands of fibrin on the epicardium. Strands of fibrin were seen floating in the fluid between the epicardium and pericardium. Ultrasonography of Angus cow abdomen revealed reticular changes typical of traumatic reticuloperitonitis, such as reduced motility and echogenic deposits.

Although neither radiography nor ultrasonography alone can achieve a complete definitive diagnosis of traumatic pericarditis, the two tools complement each other (Sharma MC, Kumar P., 2006).

As differential diagnosis in cattle with distension of the jugular veins and tachycardia, right-sided heart failure attributable to valvular endocarditis, cardiomyopathy, cardiac leucosis or other causes, must be considered (Grunder, 2002). Distension of the jugular veins without signs of right-sided cardiac insufficiency may be caused by obstruction or compression of the cranial vena cava by a thrombus or thoracic mass. Based on these poor results, under normal circumstances, cattle with traumatic pericarditis should be euthanized as quickly as possible. Treatment (pericardiocentesis and pericardial lavage, pericardiotomy associated with pericardial lavages, or pericardiostomy with fifth rib resection) should be attempted only in a valuable animal or in an animal carrying a high value embryo.

CONCLUSIONS

Clinical examination alone do not allow a definitive diagnosis of pericarditis, because all of the typical signs, which include muffled heart sounds, tachycardia, pericardial sounds, oedema and distension of the jugular veins, may not be present in every case. In doubtful cases, ultrasonography of the heart and reticulum are indicated. In Angus cow with pericarditis and reticuloperitonitis the prognosis was poor, and pericardiocentesis or pericardiotomy were inadequate methods of treatment, thus euthanasia was indicated.

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APPLICATIONS OF CLINOPTILOLITE IN VETERINARY MEDICINE AND ANIMAL HUSBANDRY

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Abstract: Zeolites are compounds of either natural or synthetic origin and have been employed successfully in a number of industries, including veterinary medicine and animal husbandry. Clinoptilolite is the form of naturally occurring zeolite that has undergone the most scientific study out of the 140 various varieties and is also used the most as a component in animal feed. Natural clay minerals and zeolites are administered with encouraging results due to their detoxifying, antioxidant, hemostatic, anti-diarrheal, and immunostimulant properties. In this paper, the characteristics and possible applications of clinoptilolite as an animal feed additive in order to prevent and/or treat particular diseases in dairy cows will be discussed. Natural and manufactured zeolites have been used as feed additives in cow nutrition, primarily to reduce the absorption of mycotoxins through the gastrointestinal tract, improve general health status, and increase overall animal performance. Clinoptilolite is marketed as a feed additive that helps maintain and improve the fertility, well-being, and productivity of cattle

Key words: clinoptilolite, dairy cow, feed additive, immunostimulant.

INTRODUCTION

According to the International Zeolite Association (IZA), there are 67 different types of natural zeolites, each of which has its own three-letter code. The Commission Implementing Regulation (EU) No. 651/2013 authorizes sedimentary-derived clinoptilolite, which is often the most popular natural zeolite, as a feed ingredient for all animal species.

Clinoptilolite is a sodium aluminosilicate that is classified and generally recognized as safe in the United States (Code of Federal Regulations CFR, Title 21, Section 182.2727). In the European Community is registered as a food additive with DIN code 53 770 (Marc S., Tulcan C., 2019).

Zeolites may be used in animal nutrition as feed additives, primarily to reduce the gastrointestinal absorption of mycotoxins. In newborn calves, they may be used as passive immunity enhancers during the colostrum period and also, to improve the health status and growth performance of animals.

In companion animals, zeolites have been used and as an adjuvant in anticancer treatment, with a promising potential outcome, according to published literature data worldwide (Marc S., Tulcan C., 2019).

Clinoptilolite is particularly significant since it might also reduce mastitis problems in dairy cattle, in addition to increasing milk yield and feed effectiveness. According to Deniz Alic Ural's 2014 study (Alic Ural D., 2014), milk production and somatic cell count were positively impacted in dairy cows receiving clinoptilolite supplementation at a rate of 3% (w/v) when it was added to the feed ratio (Benić M. *et al*, 2018).

Additionally, it has just been given EU authorization for use as a mycotoxin binder in veterinary medicine feed additives, specifically in the cattle industry (Benić M. *et al*, 2018; Valpotić H. *et al*. 2017; Valpotić H. *et al*. 2018).

This paper reviews the most important properties of clinoptilolite regarding their use in animal husbandry and veterinary medicine, as well as their potential in different areas, emphasizing the necessity of further involvement by scientists from various backgrounds for future studies regarding the applications of clinoptilolite.

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METHODS

Natural or artificial Zeolites are crystals composed of a microporous aluminosilicate skeleton containing alkaline and alkaline earth cations and a free, limitless, three-dimensional structure.

These materials have specific chemical and physical properties. They are distinguished by their ability to reversibly lose and gain water, to absorb substances with a sufficient cross-sectional diameter (adsorption property), and to remove their cations from their environment with cations such as $Ca2^{+}$, Mg^{2+}, K^{+}, and $NH4^{+}$ without significant structural changes.

Zeolite has recently been approved by the European Union as an ingredient in farm animal feed, with a maximum dry matter inclusion score of 2 percent (European Commission Order, 2005) (Khachlouf K. *et al*, 2018).

In veterinary medicine, it may act as a detoxifier, antioxidant, hepatic, antidiarrheal, antioxidant, growth stimulant, and immunostimulant agent (Valpotić H. *et al.* 2018). Zeolites may enhance animal health through several mechanisms, including direct positive effects on digestive tract morphology, function and microbial flora, delivery of metabolically active ions, improvement of nutritional status, enhancement of immunity, decontamination of food and drinking water from toxins (Bacakova L. *et al*, 2018). One of zeolites' best-known efficacy is detoxification, which is highly necessary for the removal of toxic substances from both the body and the environment.

Two zeolite properties are critical for detoxification: the exchange of ions and the capacity for adsorption. Because of this, zeolite is successful in adsorbing heavy metals like lead, silver, arsenic, cadmium, ammonia, and radioactive substances including phenols, pesticides, and mycotoxins (Eisenwagen S., Pavelić K., 2020; Pavelić S.K. *et al,* 2018).

Figure 1 shows the main properties of clinoptilolite:

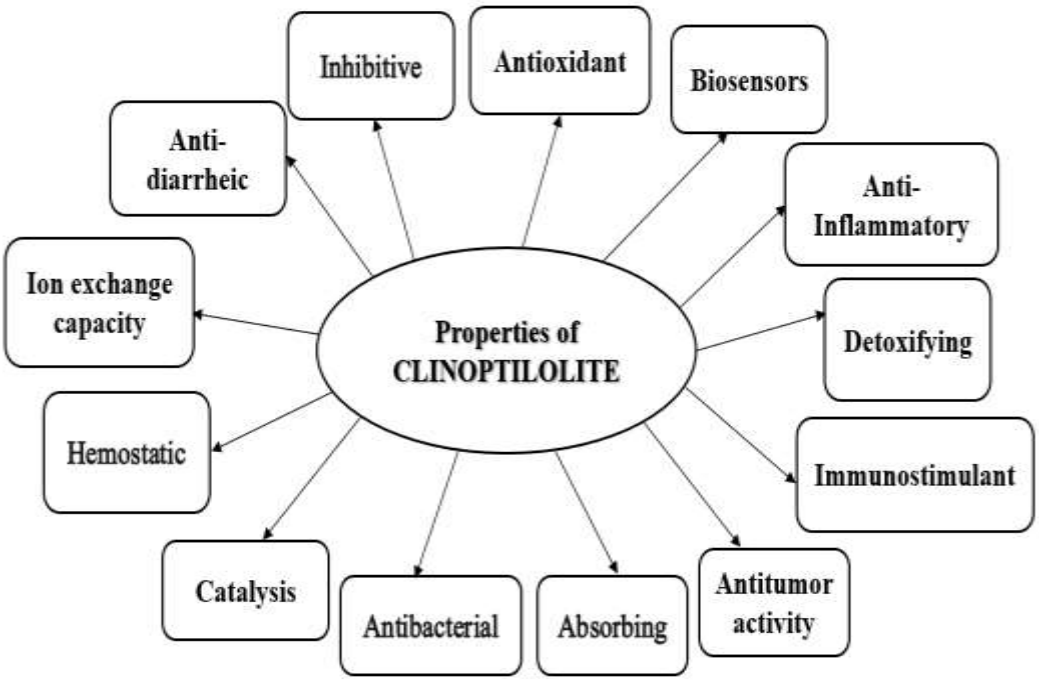

Figure 1 The main properties of clinoptilolite

DISCUSSIONS

Application of clinoptilolite

Clinoptilolite is recognized as a nanoporous material having a wide range of possible applications. Due to its excellent characteristics, particularly its porosity, specific weight, and adsorption (Samardzioska T., Jovanovski M., 2017), it can be used as a building stone in the construction industry and may also serve as a potential slow-release carrier for herbicides, insecticides and other organic compounds, protecting the environment from chemicals in the process (Marc S., Tulcan C., 2019). Figure 2 depicts the primary applications of clinoptilolite.

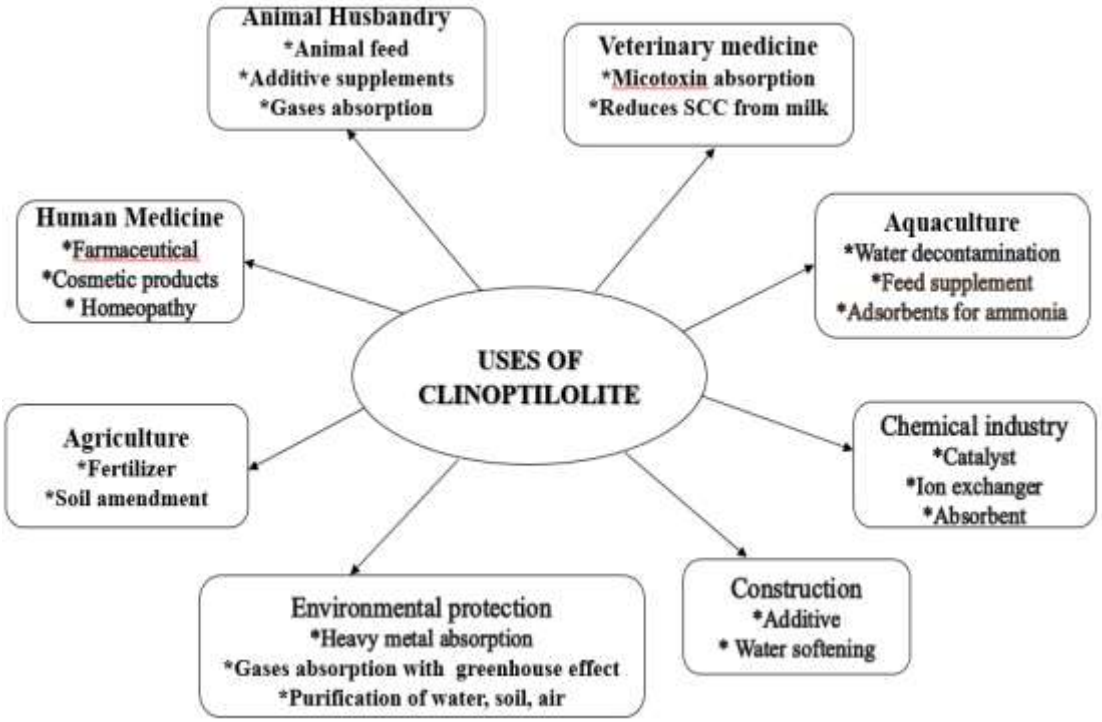

Figure 2 The main applications of clinoptilolite

Applications in veterinary medicine and animal husbandry

Clinoptilolite is considered a nano porous material with tremendous potential for use in veterinary medicine, especially in livestock production. Unsurprisingly, a large amount of work is underway to test novel approaches regarding the application of clinoptilolite in dairy cows (Valpotić H. *et al*, 2018). Clinoptilolite is the most commonly scientifically studied material among the 140 forms of natural zeolites and is best established as a zootechnical and biomedical feed ingredient commonly used as a candidate agent for replacing antibiotics in farm animal nutrition (Đuričić D. *et al*, 2017).

Natural and manufactured zeolites have mostly been employed in animal husbandry to increase production. Ammonia binding, decreasing the toxic effects of ammonia produced by intestinal microbial activity, reducing the passage rate of digesta through the intestines and improving nutrient utilization, increased pancreatic enzyme activity-favorable effect on feed components hydrolysis over a wider pH range, improved energy, and protein retention are some of the proposed applications.

Elimination of mycotoxin growth inhibitory factors is one of the mechanisms proposed for achieving the increase in productive performance in animals. Due to its detoxifying/decontaminating qualities and capacity to act as reducers of heavy metals, organic pollutants, radionuclides, and antibiotics, clinoptilolite is frequently used by livestock farmers as a feed additive for beef cattle, dairy cows, sheep, goats, pigs, poultry (broilers and egg production), rabbits, and turkeys. It is already known that adding clinoptilolite to the diet of pigs, poultry, and cattle improves their ability to acquire weight and raises feed conversion ratios.

Clinoptilolite functions as a binder of mycotoxins, blocking toxins that may be aggressive. It also helps to control aflatoxins in animal feed, thus lowering mortality from digestive stress and reducing the need for antibiotics. Clinoptilolite absorbs other toxins produced in the feed by molds and microscopic parasites and enhances food absorption by animals. The capacity of clinoptilolite to absorb aflatoxins that contaminate feed and improve the health of cattle has led to its use in this area.

There are countless reviews regarding mycotoxin binders, which present the advantages and the adsorption ability of the zeolites (Avantaggiato G. *et al*, 2005, Ariton A.M. *et al*, 2020). In the event of mycotoxicosis, zeolites may bind polar toxins such as aflatoxins, a feature confirmed in many types of research (Ariton A.M. *et al*, 2020).

Scientists have found that non-nutritional adsorbent zeolite has no harmful effect on lactating cows. Parameters are competitive when the degree

of inclusion does not go above 400 g /cow/ week. Studies carried out in the last decades have shown a high potency of clinoptilolite, *in vitro* and *in vivo* for various medical applications.

Clinoptilolite has been commonly used as an additive to animal feed or for the removal of ammonia in animal manure (Pavelić S.K. *et al*, 2018). The long-term dietary treatment of clinoptilolite to dairy calves increased milk production significantly (Katsoulos P.D. *et al*, 2005), without having any negative effects on serum concentrations of fat-soluble vitamins, specific trace minerals, or macro elements (Katsoulos P.D. *et al*, 2006). The animals' energy status was enhanced by the addition of clinoptilolite in feed during late pregnancy. Furthermore, the prevalence of metabolic disorders like ketosis was significantly decreased (Katsoulos P.D. *et al*, 2005; Katsoulos P.D. *et al*, 2006). Recently, Duričić and collaborators, (2017) evaluated the effects of dietary clinoptilolite, a novel vibro-activated and micronized modification of the natural mineral, in lactating dairy cows, on milk composition, somatic cells and the incidence of subclinical mastitis from the third to the seventh month of gestation (Đuričić D. *et al*, 2017).

The authors concluded that this predictive and beneficial consequence of clinoptilolite-supplementation may be due to its antibacterial, detoxifying, and immunostimulant impact on the incidence of subclinical mastitis and general udder health in dairy cows (Benić M. *et al*, 2018). An examination of the chemical composition and somatic cells count of milk from dairy cows, as well as a decline in the prevalence of milk fever in cows, allowed researchers to detect the favorable effects of dietary zeolites (Alic Ural D., 2014; Jorgensen R.J., Theilgaard P., 2014). Given the well-known chemical characteristics of zeolites (Pavelic K.M. et al, 2002) positive effects on health and dairy cow productivity are anticipated and demonstrated (Bosi P. *et al*, 2002). According to Valpoti and collaborators in 2017, clinoptilolite is believed to improve the health, fertility, and milk production of dairy cows by modulating their metabolic, endocrine, and antioxidant state (Valpotić H. *et al, 2018*).

In 2014, Alic Ural discovered that giving dairy cows feed containing 3% clinoptilolite enhanced milk yield and lowered the somatic cells count (Alic Ural D., 2014; Đuričić D. *et al, 2017*). The majority of research findings point to the possibility that adding clinoptilolite may increase milk production. In addition, various management techniques for clinoptilolite food supplements in dairy farms must be used with the utmost caution (Alic Ural D., 2014).

Since the content of milk fat was statistically different in the third and fourth samplings compared to the first sampling in the control group, whereas this difference was only seen between the first and third samplings in the clinoptilolite-fed cows, the chemical composition of milk was found to be more stable in this study (Đuričić D. *et al*, 2017).

Clinoptilolite shows properties, in particular, to reversible ion exchange and adsorption capacity of gases (Pavelić S.K. *et al., 2018*). Natural zeolites may absorb CO, CO_2, SO_2, H_2S, NH_3, HCHO, Ar, O_2, N_2, H_2O, He, H_2, Kr, Xe, CH_3OH, and many other gasses, and therefore can be used to capture or regulate odors. Such minerals are also used in intensive animal husbandry barns, substantially reducing the ammonia and H_2S content, which causes unwanted odors. The high absorption ability of ammonia makes it a very effective natural mean of controlling high levels of this gas, generated by animals, in farms.

This may be used in filtration systems or simply spread across the water surface, as it is absolutely harmless to aquatic life. Food crops that grow in soil containing high concentrations of Pb, Cd and Cu may also benefit by the zeolite absorption ability (Laurino C., Palmieri B., 2015). Such a result may be explained by the moderation of stressful events that occur during these times since cows are more susceptible to metabolic imbalance and environmental negative impacts during these times, which increases their vulnerability to intramammary infections and immunosuppression (Đuričić D. *et al*, 2017).

CONCLUSIONS

Zeolites can be used in animal nutrition as additives, primarily to reduce the gastrointestinal absorption of mycotoxins; In newborn calves, they can be used as enhancers of passive immunity during the colostral period. Studies have shown that the use of clinoptilolite as a feed additive in cattle can have a significant contribution to the prevention and treatment of certain diseases.

The use of natural zeolites in the feeding of dairy cows during the pre-calving, and post-calving periods and in the first part of lactation plays the role of promoter of productive performances. In this context, it is estimated that zeolites will play a more important role in food safety and agricultural practices in the near future.

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NON-DESTRUCTIVE METHODS OF MILK QUALITY ASSESSMENT

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Abstract
Monitoring the quality of raw cow milk is essential to maintain food safety and human health. To guarantee the quality and safety of milk, easy-to-use non-destructive analysis methods are available. Non-destructive methods have made progress, being useful for obtaining quantitative and qualitative data, destroying the sample, and offering several advantages, such as high sensitivity, adequate response time, and minimal sample preparation. Conventional methods are laborious, destructive, toxic, and time-consuming, require fully equipped laboratories and skilled personnel, extensive sample preparation, and are not suitable for real-time continuous monitoring of milk quality. The rapid development of non-destructive methods for the evaluation of milk quality consists of applications based on imaging, near-infrared spectroscopy, conductivity, biosensors, and ultrasound. This paper presents different non-destructive methods applied to determine the physicochemical parameters of raw milk (fats, proteins, lactose, casein, dry matter, acidity, density), the number of somatic cells, and the microbiological load.

Key words: raw milk, non-destructive methods, physico-chemical parameters.

INTRODUCTION

The quality of raw milk has always been the subject of research at the national and international levels, through different analysis techniques, having a direct influence on health and food safety (Oarga A.C.D, 2010).

The determinations made in Romania regarding the physico-chemical quality of raw milk are based on globally standardized analysis methods. At the national level, the physico-chemical quality parameters of raw milk are regulated by STAS 2418:2008-Full raw milk. Potentially non-destructive methods, such as imaging analysis, NIR spectroscopy, NMRI, ultrasound, X-ray imaging, and biosensors may supplement or replace many of the traditional time-consuming destructive methods (Narsaiah K., *et al*, *2012*). Over the past 30 years, Near Infrared Spectroscopy (NIR) has proven to be one of the most influential and advanced tools for continuous monitoring and quality control of dairy products. There is a growing demand from consumers in the food industry sector to have fast-measuring devices that allow the characterization of raw materials or food. Conventional chemical methods cannot determine the parameters of dairy products in a short time (Růžičková J. *et al., 2006*; Bondoc I., Șindilar E.V., 2002). The identification of microorganisms in the dairy industry relies mainly on conventional standard methods such as plate count and culturing, microscopy, flow cytometry, or on advanced immunological techniques (e.g. biochemical kits, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) (Mandal *et al, 2011*; *Law et al, 2015*; Zhao *et al, 2014*). Commonly used as the gold standard, conventional plate count and culture procedures are very sensitive (10-100 CFU/ml), affordable, and capable of providing qualitative and quantitative data on the quantity and kind of microorganisms. However, they are labor-intensive, time-consuming (the time needed for pathogen identification often takes more than a week) (Zhao *et al, 2014*), and unsuitable for real-time, on-site applications. Since they can reduce the amount of time needed for detection following enrichment to as little as 1-3 hours, immunological techniques using robotic and automated ELISAs are frequently used. However, the findings can only be available in 1-2 days due to sampling pre-processing and enrichment stages. PCR-based methods enable the identification of bacteria through their genetic makeup and do not necessitate a step involving bacterial culture (Law JW-F. *et al, 2015*; Mortari A., Lorenzelli L., 2014). Although PCRs take between 30 and 90 minutes to complete, the entire process including sample

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preparation, DNA extraction, and amplicon analysis—can take from 6 to 8 up to 48 hours.

The inability of PCR-based approaches to differentiate between living and dead cells is a significant flaw that could result in overestimation and false-positive results. This paper presents different non-destructive methods applied to determine the physicochemical parameters of raw milk, the number of somatic cells and the microbiological load.

MATERIAL AND METHOD

Materials: milk, combiscope kit (decon, triton, coloration solution), Neogen kit, Clear Milk test plates.

Methods

The evaluation of the density of cow's milk was carried out with the help of the analytical balance to which a specific determination kit was attached.

Preparation of the sample to be analyzed: Carefully pour the milk into a 250 ml Berzelius glass, held in an inclined position, to avoid the formation of foam or air bubbles. After balancing, insert the Berzelius glass with milk inside the balance. The kit for determining the density is easily inserted, and then the obtained value is displayed on the screen of the analytical balance *(Figure 2).*

Evaluation of protein content by the Dumas Method - The Dumas method consists in burning the sample of known mass in a high-temperature range of 800-900 °C in the presence of oxygen, nitrogen, and helium. The gases are then passed through special columns that absorb carbon dioxide and water.

Preparation of the sample to be analyzed: Weigh the sample on the analytical balance. For the analysis of liquid products, an absorbent/hygroscopic substance is used, in which a maximum of 100 mg of milk is added with a pipette. Weighed samples are folded in tin foil and added to a numbered rotor (weigh 3-5 samples on average). After every 4 minutes, the result of each sample expressed in total nitrogen content is obtained, and by multiplying it with the correction factor of cow's milk 6.38, the protein content is obtained (*Figure 1*).

Figure 1 Rotor of the Automatic Analyzer – Dumatherm

The evaluation of the physico-chemical parameters and the number of somatic cells - may be done with the Combiscope type analyzer consisting of Lacto-Scope FTIR and Soma Scope - MK2. The Combiscop is a fast and complex analyzer whose principle of analysis is based on infrared measurement. It is composed of two parts: LactoScope FTIR used for measuring the physicochemical parameters of milk and SomaScope used for counting somatic cells in raw milk.

Preparation of the sample to be analyzed: - the samples are inserted into Falcon-type tubes with a capacity of 50 ml. On the label, data regarding the registration number of the animal from which the respective sample was collected and the date of collection are mentioned. Subsequently, the samples are heated beforehand at 37 ^{0}C in a water bath, then placed on a special rack. The rake with samples moves on the band of the device and the syringe draws a quantity of 1 mL of milk from each Falcon tube.

The sample is analyzed for 1 minute, then the results are displayed on the computer software.

LactoScope FTIR - uses special kits both for determining the physico-chemical composition of milk (basic compounds: protein, fat, dry matter, lactose, casein), but also for other additional parameters (urea, free fatty acids, density). ***Somascope*** - Specific kits are used to determine the total number of somatic cells/mL of milk.

Evaluation of the total number of germs (TVC) with *Soleris*® *System (Neogen)* - this technique

consists of determining pH, metabolic activity during growth of the microorganism, and other biochemical reactions within 24 hours. One mL of the milk sample is inoculated into the Soleris vial containing a selective growth medium and a pH indicator (NB-100, Total Viable Count Medium-TVC), followed by incubation for 18–24 hours at 35°C. The system consists of a single incubator with 32 independently monitored and temperature-controllable locations, with a range between 15°C and 60°C.

During testing, in less than 24 hours, the Soleris software reported positive test findings (Crivei I.C*. et al, 2022)*. The absence of detection within 24 hours was considered a negative result. In the case of positive samples, the growth curves are examined, and the visual validation of medium color change is also performed. Also, randomly, for some of the positive results, standard methods were used for confirmation.

Evaluation of the microbiological load - can be evaluated with Clear Milk test plates made of - Three-section Petri dish with chromogenic agars, a tube for milk collection inoculation wand for transferring milk to the Petri dish, napkin for disinfecting the teat before sampling. The milk sample is taken in a sterile test tube; the milk is applied with the inoculation wand to the surface of all three agars of the Petri dish; The plate is incubated for 22-26 hours at 37.5°C and the species of the pathogen are assessed according to the atlas https://www.clearmilk.cz/en

RESULTS AND DISCUSSIONS

The analyzed milk was collected weekly from the collection tank of the farm S.C.D.C.B. Dancu, Iași, and the processing of the analyzed matrix was carried out periodically. The physico-chemical parameters and the microbiological load (density, acidity, fat, protein, lactose, dry matter, NCS, NTG) were evaluated, applying different non-destructive methods. The evaluation of the density of cow's milk was carried out with the help of the analytical balance to which a specific determination kit was attached *(Figure 2)*, the results obtained falling within the range of 1.028 - 1.031 g/cm^3, with a general average of 1.030±0.001 and a coefficient of variation of 0.11 %. The obtained results were also verified by the classic method (with thermolactodensimeter), the results being similar. The density of milk is also given by the automatic analyzer - CombiScope 600/300 FTIR System.

Figure 2 Specific kit for measuring milk density

Determination of protein content by the Dumas method

The first method developed for determining the nitrogen content of milk, including food, was developed in 1826 by Jean-Baptiste Dumas. The instrument must be carefully standardized using a compound of known nitrogen content (eg EDTA, Glycin used to calibrate the thermal conductivity detector). The Dumas method is easy to use and fully automatic *(Figure 3).* It was developed as a much faster method than the Kjeldahl method, and the determination of total nitrogen content can be determined in a few

minutes, compared to 6 hours or even more in the case of the Kjeldahl method. It also does not use toxic substances or catalysts. As with the Kjeldahl method, it does not provide a measurement of protein, as it records non-protein nitrogen, and different correction factors are required depending on the type of matrix analyzed, as they have different amino acid sequences.

Figure 3 Illustration of the Dumas Analyzer

The evaluation of the physico-chemical parameters and the number of somatic cells - can be done with the Combiscop Analyzer consisting of Lacto-Scope FTIR and Soma Scope - MK2 *(Figure 4).*

Establishing the health status of the udder, as an indicator of the quality of the raw milk, was achieved by determining the number of somatic cells and the physical-chemical parameters. Following the analysis carried out in the hot and cold seasons, on the samples collected from the tank, the results were the following:

Dry matter content and fat content varied accordingly. Thus, in milk samples with an average fat of over 4.0%, the dry matter content exceeded 12.5% on average, and in the warm season, when the percentage of milk does not exceed 3.8%, the dry matter content was on average 12.05 %.

The amount of fat (total lipids) was in the range of 3.5% - 4.25%, with an average of 3.80%. In the cold season, the values were between 3.6% - 4.25%, with an average of 3.9%, a fact due to the fodder ration administered, and in the warm season, the values were between 3.5% - 3.8%, with an average of 3.7%.

The protein content of the analyzed milk samples fell within the range of 3.15% - 3.4%, with an average of 3.368±0.057%. The determination of the lactose content was carried out at Combiscope and the average values vary depending on the fat and protein content, the variation range being between 3.36% - 5.21%, with an average of 4.717±0.112%

The casein content varied according to the protein values, falling between 2.75% - 3.1%, with an average of 2.8±0.12%. Establishing the state of health of the udder, as an indicator of the quality of milk - raw material, was achieved by determining the number of somatic cells, using the FTIR CombiScope 600/300 System.

Thus, the determinations made indicated that the milk - raw material, from the experimental biobase of SCDCB Dancu, falls within the norm recommended by the EU regarding the quality of fresh milk (maximum 400 000 cells/mL), having values between 249 000 – 361200 cells/mL, with an average value of 305 100 cells/mL milk – raw material.

Figure 4 Automated Analyzer – CombiScope 600/300 FTIR System (LactoScope FTIR 600/300 and SomaScope LFC 600/300)

Evaluation of the total number of germs (TVC) with *Soleris® System (Neogen*)

Determination of the quality of raw milk in terms of its degree of sanitation was carried out by determining the total number of germs (TVC), using the rapid microbial detection system Soleris® System (Neogen) *(Figure 5; 6).* The determination of the quality of the milk in terms of the degree of sanitation was achieved by determining the total number of germs. The maximum duration of sample analysis can be up to 12 hours, depending on the microbial load of the raw milk samples.

Establishing the total number of germs by applying this technique consists in determining the pH, the metabolic activity during the growth of the microbial load, and other biochemical reactions, within 24 hours. Following the testing of milk samples from the experimental biobased of

S.C.D.C.B. Dancu found that the milk corresponds from a hygienic point of view, falling within the norm recommended by the EU (TVC < 100.000 CFU/mL), with values between 29.000 – 62.000 CFU/mL, with an average value of 45.500 CFU/mL milk - raw material. Thus, compared to the serial dilution method (classical method), this rapid technique offers the advantages of reduced analysis time and costs.

Figure 5 Graphical representation of the detection curve of the total number of germs generated in real time

Figure 6 System for determining the total number of germs (TVC) – SOLERIS

Evaluation of the microbiological load – The Clear Milk test plates were used for the rapid detection of pathogens in milk. The presence of more than one colony of major pathogens such as *Streptococcus agalactia, and Staphylococcus aureus* indicates a positive result. The presence of more than five colonies of other pathogens such as *Streptococcus uberis*, and *Streptococcus dysgalactia* is likewise, an indicator of positive results.

Figure 7 shows a plate inoculated with milk collected from a cow with clinical mammity in which a high number of somatic cells (6 502 000 cells/mL) and the presence of Streptococcus dysgalactia pathogens were identified.

This type of on-farm culturing systems enables veterinarians to establish a proper treatment approach in order to ensure a the recovery of the mammary gland and prevent antibiotic resistance.

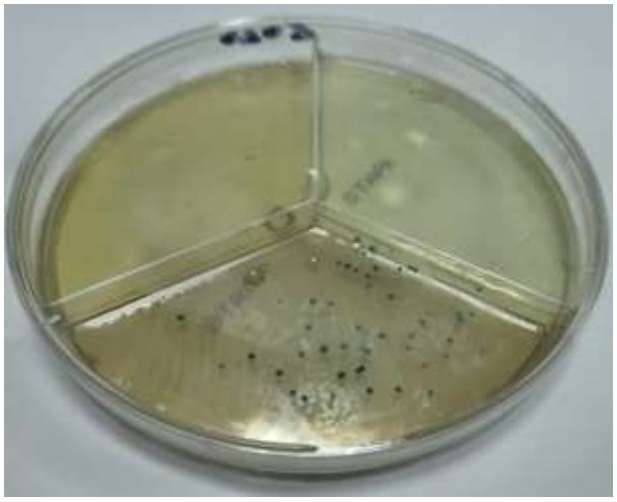

Figure 7 Milk sample from cow with mastitis cultured on Clear milk test plate – Streptococcus dysgalactia colonies visible

CONCLUSIONS

NIR spectral techniques can provide results with a high degree of accuracy in a short time, offering advantages in terms of working time, price, and human resources.

The development and use of non-destructive or portable instruments follow the idea of green analytical chemistry which involves a drastic reduction in costs, analytical steps, sample pre-treatment, energy, and reagents, and for this reason, the use of these devices should be promoted to help cattle farmers to monitor milk quality and be able to have low costs in this regard.

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https://www.clearmilk.cz/en.

PREVALENCE OF TICK INFESTATION IN DOGS FROM IASI AREA (ROMANIA)

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Abstract

Dogs and cats play an important role in the spread of tick species and in the transmission of pathogens. *Dermacentor reticulatus* occurs in areas by varied types of climate, vegetation and a high availability of potential hosts, while *Ixodes ricinus* follows a similar seasonal pattern each year. This study was carried out between 2017 and 2021, where we examined 189 canine patients of different breeds, aged between 2 months and 18 years. We collected 435 ticks which were placed individually according to the animal in eppendorf tubes in ethyl alcohol and numbered, until morphological identification. The collected ticks were predominantly adults with different feeding status, belonging to the species: *Ixodes ricinus* (41.6%), *Dermacentor reticulatus* (57.7%) and *Rhipicephalus sanguineus* complex (0.69%). *Dermacentor reticulatus* was predominant, with two peaks of activity in spring and autumn. We also found 2 specimens of *D. reticulatus* males, in January, outside the normal period of activity. The second species identified, *Ixodes ricinus*, shows three peaks of activity, having the most intense period of activity in the spring months. The second peak is observed in summer, and the third in autumn. The prevalence of the species *Rhipicephalus sanguineus* complex is low, of only 0.69% of the total ticks collected from dogs.The aim of this study was to determine the prevalence of tick infestations collected from canine patients, in order to assess a possible change in the activity periods of the species.

Key words: Dermacentor; seasonality; Ixodes; epidemiology;

Pets play an important role in the spread of native tick species and the transmission of pathogens to other animals and humans (Buczek et al., 2021). Dogs are usually infested by adult ticks (Földvari et al., 2005; Dutto et al., 2009), in smaller numbers by nymphs (Földvari et al., 2005; Dutto et al., 2009; Estrada-Peña et al., 2017) and sporadically by larvae (Dutto et al., 2009; Hansford et al., 2016; Manilla, 1998; Kumsa et al., 2011; Saleh et al., 2019). The most common body sites infested with ticks are the head (ear area, eyes and muzzle), legs and neck (Dutto et al., 2009; Lorusso et al., 2010; Wright et al., 2018), and the frequency of tick attachment is associated with dog breed and temperament (Lorusso et al., 2010).

The ticks that attach to dogs differ depending on the seasonal activity of the species involved. These activity patterns are supported by previous studies examining the seasonal activity of ticks collected from hosts or natural habitats (Lengauer et al., 2006; Dautel et al., 2008; Kohn et al., 2011; Cull et al., 2017).

Infestation of dogs with ticks, especially the species *Dermacentor reticulatus*, has increased in recent years in some parts of Europe (Heile et al., 2006; Beck et al., 2014). Warmer winters, but also extended autumn and spring seasons, cause the expansion of tick distribution at higher altitudes but also in northern latitudes (Beugnet et al., 2013; Dantas-Torres, 2015). *D. reticulatus* may be active in January and February, shortly after the snow cover disappears (Karbowiak, 2014). Also, the fat bodies present in some *Dermacentor reticulatus* ticks are used to survive the winter (Zajac et al., 2020).

Dermacentor reticulatus occurs in areas characterized by varied types of climates, vegetation and a high availability of potential hosts, therefore presenting ecological plasticity and high adaptability to periodic unfavorable conditions (Zajac et al., 2020). This species has been identified in western Siberia as well as in the steppes of Ukraine and Russia of the European continent, where the temperate climate is characterized by extremely high annual temperature values and low annual precipitation (Rubel et al., 2016; Miller, 2019).

In endemic areas of central Europe, *Dermacentor reticulatus* shows a frequency comparable to *Ixodes ricinus* in dogs, and represents 45% of ticks in Germany (Beck et al., 2014) and 49% of ticks in Hungary (Földvari et al., 2005).

Ticks belonging to the genera *Ixodes* and *Rhipicephalus* are the most widespread in Italy,

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according to Maurelli et al. (2018). The authors of the study identified a prevalence of the species *Rhipicephalus sanguineus* of 27.5% in dogs from Northern Italy and 36.1% in those from the central and southern regions, while the prevalence of the species *Ixodes ricinus* was 25.6% in the regions of Northern Italy and only 10.8% in dogs from the center and south of the country. Only a few dogs were infested with *Dermacentor* spp. (0.6%).

In Romania, Raileanu et al. (2018) collected ticks from 13 sites distributed in the urban area of Iasi, identifying three species of ticks: *Ixodes ricinus* with a prevalence of 95.3%, *Haemaphysalis punctata* – 3.9% and *Dermacentor reticulatus* – 0.8%. Similar results were found by Pavel in his doctoral thesis research (2014) where the prevalence of the species *Ixodes ricinus* was the highest (92.39%), followed by *Haemaphysalis punctata* (6.94%) and *Dermacentor reticulatus* (0.59%).

Cull et al. (2018) consider that *Ixodes ricinus* follows a similar seasonal pattern each year, although there may be a variation in peak activity in May, June and July depending on the year. Also, they observed an increase of activity in the autumn months.

Predictive spatiotemporal patterns (eg. type of vegetation, climatic conditions or wildlife population density) of tick abundance may be important to reduce the incidence of tick-borne diseases (Hartemink et al., 2015; Sgroi et al., 2022). Also, the relationship between temperature and tick developmental stages is not linear for ixodid ticks (Hollingsworth et al., 2015).

The aim of this study was to determine the prevalence of ticks collected from canine patients, in order to assess a possible change in the activity periods of the species.

MATERIAL AND METHOD

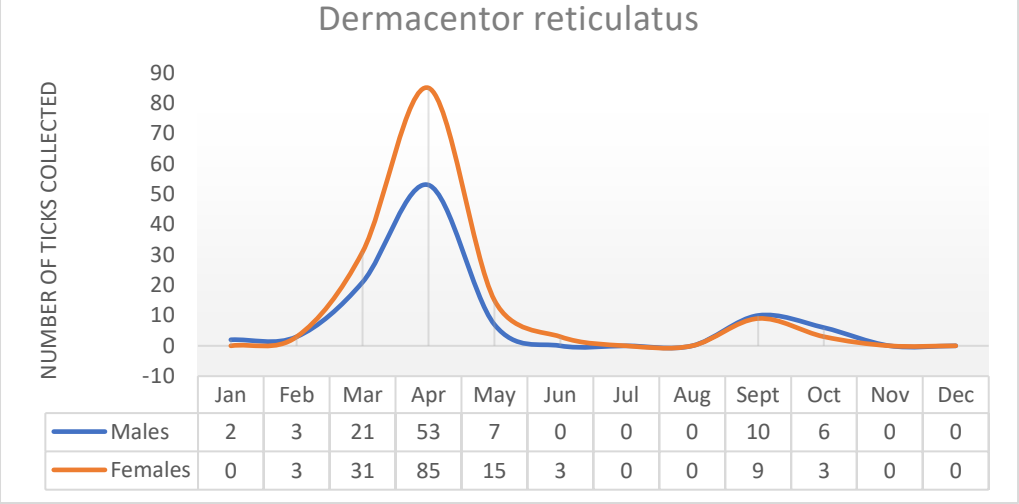
The study was carried out between 2017 and 2021 within the Parasitic Diseases Clinic and the Emergency Department of the Faculty of Veterinary Medicine Iași. During this period, 411 dogs of different breeds, aged between 2 months and 18 years, which were presented for various paraclinical investigations, were examined. All the ticks were removed with a special devices, which were placed individually according to the animal in eppendorf tubes (2ml–5ml), in ethyl alcohol with a concentration of 70% and numbered, until morphological identification.

After the general clinical examination, we found the presence of ticks attached to a number of 189 canine patients (45.99%).

The morphological identification was carried out within the Department of Parasitic Diseases of the Faculty of Veterinary Medicine Iasi, using standard taxonomic keys (Estrada-Pena et al., 2004; Dantas-Torres et al., 2013), as well as the Zeiss Stemi 305 stereomicroscope.

RESULTS AND DISCUSSIONS

Following the centralization of the data, between 2017 and 2021, 411 dogs of different breeds and ages were examined, and a number of 435 ticks were identified on 189 dogs (45.99%),



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Males	2	3	21	53	7	0	0	0	10	6	0	0
Females	0	3	31	85	15	3	0	0	9	3	0	0

Figure 1 - Prevalence of *Dermacentor reticulatus* species

following the general clinical examination. The collected ticks were predominantly adults (99.54%), with different feeding status. This aspect was also observed by other authors (Földvari et al., 2005; Eichenberger et al., 2015; Dumitrache et al., 2014), where more than 90% of ticks collected from dogs

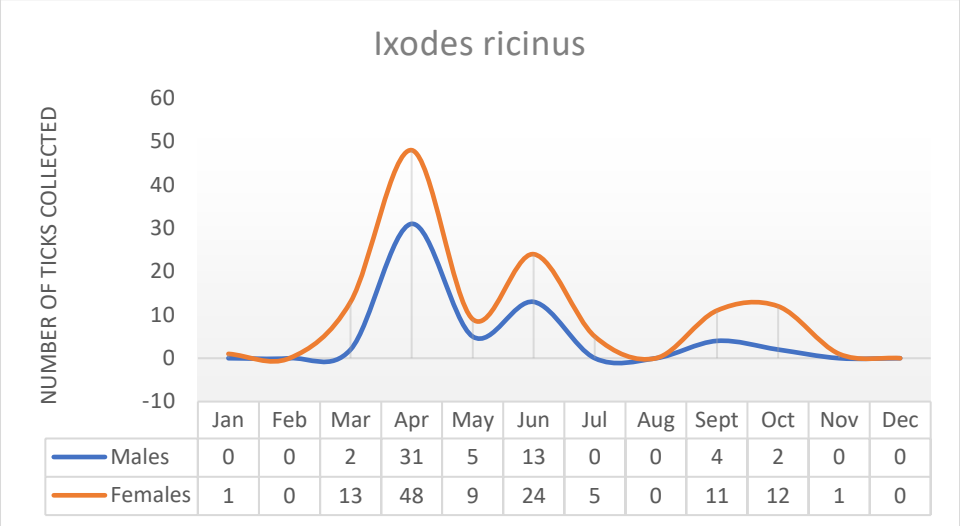
were adults. The explanation of these results is given by the impossibility of macroscopic observation of nymphs and larvae during clinical examination.

Morphologically, we identified three species of ticks also exposed in: *Ixodes ricinus* (41.6%; 181/435), *Dermacentor reticulatus* (57.7%; 251/435) and *Rhipicephalus sanguineus* complex (0.69%; 3/435). Dogs can be infested with extremely many species of ticks. One such example was reported in Russia, where Livanova et al. (2018) detected 11 species of ticks, from four genera: *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Haemaphysalis*. In southeastern Romania, Dumitrache et al. (2014) identified six species of ticks attached to dogs: the most common species was *R. rossicus* (95.6%), followed by *D. reticulatus* (3.2%), *I. ricinus* (0.5%), *H. marginatum* (0.3%), *R. sanguineus* s.l. (0.2%) and *I. crenulatus* (0.1%). Abdullah et al. (2016) conducted a study on the species of ticks infesting dogs in the United Kingdom, identifying in dogs that had not left the country in the last two weeks the following species: *Ixodes ricinus* (89.2%), *Ixodes hexagonus* (9.8%), *Ixodes canisuga* (0.78%), *Haemaphisalys punctata* (0.05%) and *Dermacentor reticulatus* (0.17%). Dogs that have left the country in the last two weeks were infested with one of the following species: *Ixodes ricinus* (67.4%), *Dermacentor variabilis* (2.3%) and *Rhipicephalus sanguineus* (30.2%), thus amplifying concerns about the importation of ticks through livestock company traveling to endemic areas.

According to the results obtained, reported in *Figure 1*, *Dermacentor reticulatus* was the dominant species, with two peaks of activity in the spring, in the months of March (52 specimens: 21 males; 31 females), April (138 specimens: 53 males; 85 females) and autumn – September (19 specimens: 10 males; 9 females), October (9 specimens: 6 males; 3 females). Duscher et al. (2013) observed the same frequency of activity of *Dermacentor reticulatus*, in spring and late autumn. Also, in this study we found 2 specimens of *D. reticulatus* males, in January, outside the normal period of activity. Similar results were also published in the study by Mierzejewska et al. (2015): males of *D. reticulatus* constituted the majority of ticks collected from dogs during winter, and Dautel et al. (2008) collected some unfed adult ticks from vegetation in January and February. The most common tick species identified in dogs in Russia was *Dermacentor reticulatus*, with a prevalence of 40.7%, followed by the species *Ixodes ricinus* (12.1%) and *R. sanguineus* (11.3%) (Livanova et al., 2018).

Ixodes ricinus is more active than *D. reticulatus* from April to October, and *Dermacentor reticulatus* was more frequently identified than *I. ricinus* from January to March, and from September to November (Beck et al., 2014), these data also being consistent with the results of our study. According to the study by Pavel et al. (2014) in Iasi, the seasonal activity of unfed ticks presented a bimodal pattern, with peaks of activity in spring and autumn, as follows: *Dermacentor reticulatus* recorded the first peak in March, decreasing in the following months and ceasing activity during the summer and the species *Ixodes ricinus* showed maximum activity in April and May.

The activity of the *Ixodes ricinus* species identified in our study shows three peaks of activity, with a downward trend in the colder months (*Figure 2*). Thus, the most intense period is identified in the spring months (March, April), followed by a decrease in May. The second peak is observed in June and the third, in September and October. In general, *Ixodes ricinus* is absent in the winter months (Mierzejewska et al., 2015), but incidentally, the presence of these species can also be observed in the months with low temperatures, as we also identified in this study, a single tick in month January. In the urban area of the city of Cluj, the activity of *Ixodes ricinus* larvae is unimodal, starting in May, while the nymphs and adults show a bimodal activity, with an increased activity observed from April to June (Borșan et al., 2020). The results obtained by Földvari et al. (2005), *Ixodes ricinus* and *Dermacentor reticulatus* were active throughout the year except July and December. In the study conducted in Northeast Germany, Beck et al. (2014) found that *Ixodes ricinus* can be found throughout the year, except for January, and the species *D. reticulatus* shows no activity in July.



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Males	0	0	2	31	5	13	0	0	4	2	0	0
Females	1	0	13	48	9	24	5	0	11	12	1	0

Figure 2 - Prevalence of *Ixodes ricinus* species

R. sanguineus is a common species in the Mediterranean region, it is frequently imported via dogs to countries in Northern or Central Europe: Great Britain (Jameson et al., 2010; Tulloch et al., 2017; Gillingham et al., 2020), Germany (Menn et al., 2010; Röhrig et al., 2011; Schäfer et al., 2019) Netherlands, Denmark, Switzerland (Buczek et al., 2021), Belgium (Claerebout et al., 2013). Less frequently, this species can also be transferred from other endemic countries, for example, the Dominican Republic (Dongus et al., 1996) or Sudan (Buczek et al., 2021).

The present study identified a low prevalence of the species *Rhipicephalus sanguineus* complex, of only 0.69% of the total ticks collected from dogs, as follows: one larva collected in April, one nymph and one female in May. *Rhipicephalus sanguineus* had a prevalence of 0.1% in the study made by Beck et al. (2014), a value close to that reported in the present study. Maurelli et al. (2018) observed a peak infestation of *Rhipicephalus sanguineus* ticks during spring and summer, being collected from dogs in all months of the year. In Ukraine, *R. sanguineus* has been identified in dogs on the coast of the Black Sea and the Sea of Azov, and occasionally it has also been reported in cats and foxes (Nebogatkin, 2013; Rogovskyy et al., 2017). In Albania, the prevalence of *R. sanguineus* species identified in dogs was 8.1%, and that of *Ixodes ricinus* species was only 0.8% (Shukullari et al., 2017). *Rhipicephalus sanguineus* s.l. was identified in dogs from Spain with the highest prevalence (53%), followed by the species *Dermacentor reticulatus* (9%), *Ixodes ricinus* (9%) and *I. hexagonus* (4%). Adults of *R. sanguineus* were observed throughout the year, but the maximum peak of activity was in March-July (Estrada-Peña et al., 2017).

Following the clinical examination of the patients, we found that most canine patients - 41.2% (78/189) were infested with one tick, 23.3% (44/189) were infested with two ticks and 20.6% (39/189) with three ticks. Also, in 2/189 (1.1%) of the patients we identified increased infestations, with more than ten ticks, as shown in the data presented in *Table 1*. The intensity of tick infestations varies between 1 and 78 ticks/dog (Földvari et al., 2005). Dumitrache et al. (2014) identified a higher prevalence of single-species infestations, which was 92.3%. The largest tick infestation observed by Eichenberger et al. (2015) was 49 ticks per dog, belonging to the species *Ixodes ricinus*, but 55.6% of the dogs included in the study were infested with a single tick. In Europe, the role of dogs in the transmission of native and exotic tick species has been investigated in Germany (Abdullah et al., 2016; Hansford et al., 2016), Sweden (Jaenson et al., 2012), Switzerland (Eichenberger et al., 2015), Hungary (Földvari et al., 2005; Földvári et al., 2007), Cyprus (Tsatsaris et al., 2016), Spain (Estrada-Peña et al., 2017) and Portugal (Dantas-Torres et al., 2017).

Humans can also host tick species following travel to exotic countries, thereby bringing in new species (Gillingham et al., 2020). The future of the planet's climate is uncertain in the long term, so the impact of climate change on biodiversity and tick-borne diseases is unpredictable. Some causes and consequences of climate can vary in time and space, some being reversible (Dantas-Torres, 2015). The geographical range of many tick species depends mainly on the biology and ecology of the specific species, as well as on biotic and abiotic factors, since each species has environmental conditions and preferred biotopes that determine their geographical

distribution and, consequently, the delimitation of risk areas for tick-borne diseases (Dutto et al., 2009).

Table 1

Prevalence of collected ticks from dogs after clinical examination

No. ticks present on the dog / Month	1	2	3	4	5	6	7	8	9	>10
Jan.	3	0	0	0	0	0	0	0	0	0
Feb.	1	1	1	0	0	0	0	0	0	0
Mar.	14	9	5	2	1	0	1	0	0	0
Apr.	**34**	**17**	**16**	**8**	**5**	**1**	**0**	**1**	**0**	**2**
May	4	2	3	0	1	1	0	0	0	0
Jun.	6	5	3	2	0	0	1	0	0	0
Jul.	4	1	0	0	0	0	0	0	0	0
Aug.	0	0	0	0	0	0	0	0	0	0
Sept.	5	6	4	0	1	0	0	0	0	0
Oct.	6	3	2	0	1	0	0	0	0	0
Nov.	1	0	0	0	0	0	0	0	0	0
Dec.	0	0	0	0	0	0	0	0	0	0
	78	**44**	**39**	**12**	**9**	**2**	**2**	**1**	**0**	**2**

Legend: nr. – number; Jan. – January; Feb. – February; Mar. – March; Apr. – April; Jun. – June; Jul. – July; Aug. – August; Sept. – September; Oct. – October; Nov. – November; Dec. – December;

Temperature and relative humidity affect ticks' rate of host search as well as survival (Lindgren et al., 2006; Ruiz-Fons et al., 2012). In Northern European urban forests, the population dynamics of larvae and nymphs belonging to the species *Ixodes ricinus* are closely related, with data in the specialist literature on synchronous activity, thus suggesting a higher abundance of ticks and consequently a higher risk of pathogens that can infect humans and domestic animals (Cayol et al., 2017).

Human behavior is also a determinant of environmental protection, human and animal health, with an example being the avoidance of tick-infested habitats by humans that could change the risk pattern of disease transmission (Dantas-Torres, 2015). Urban and sub-urban areas are increasingly affected by vector expansion (Grochowska et al., 2020; Oechslin et al., 2017) due to environmental interventions. However, green areas inside or around cities exert a positive effect on the quality of life and human health (Cetin, 2015). The COVID-19 pandemic has shown the need to make cities more livable, but also the danger of close interaction with wildlife species, thus increasing the risk of exposure to various pathogens (Bellato et al., 2021).

The effects of tick parasitism on pets and their owners can be limited by the periodic application of various protection methods recommended by veterinarians.

CONCLUSIONS

The predominant tick species collected from dogs in Iasi county, in the period 2017-2021 was *Dermacentor reticulatus* (57.7%), followed by *Ixodes ricinus* (41.6%) and *Rhipicephalus sanguineus* complex (0.69%).

All ticks in this study were feeding at the time of collection, therefore the possibility of transmitting pathogens to the host was an increased one, being an indication of pathogens present in the local community.

Also, the increased prevalence of the *Dermacentor reticulatus* species in dogs explains the numerous cases of canine babesiosis reported in the area of Iași and the identification of the *Rhipicephalus sanguineus* complex species in this area reinforces the idea of continuing investigations to identify the pathogenic role on humans and animals.

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SHOULD GLOBAL WARMING INFLUENCE THE OCCURRENCE OF CANINE DIROFILARIOSIS INFECTION IN ROMANIA?

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Abstract

In order to evaluate the prevalence of heartworm disease in the South-East of Romania, we collected blood from 40 dogs from a shelter in Galati, which were examined by several methods for the *Dirofilaria immitis* and *Dirofilaria repenes* microfilariae identification, and for the detection of *Dirofilaria immitis* antigen respectively. Eleven dogs were positive for microfilariae (27.5%), of which 3/11 were co-infections and only 1/40 had *D. repens* microfilariae detected. Following serological test, *D. immitis* antigen was detected in 30% of the investigated serum samples (12/40). The high prevalence of heartworm disease requires continuous monitoring of symptomatic dogs, but also of the asymptomatic ones from shelters or of those from veterinary clinics in endemic areas. Furthermore, the mosquito populations control in endemic areas is mandatory.

Key words: canine dirofilariosis; shelter; Galati; global warming;

Canine dirofilariosis is a potentially zoonotic disease transmitted through mosquito bites, which is produced by the nematodes *Dirofilaria immitis* and *Dirofilaria repens* (Capelli et al., 2018). The intermediate hosts (the vectors) involved in the transmission of these nematodes are *Culex*, *Anopheles* and *Aedes* genus (Mirahmadi et al., 2017; Adebayo et al., 2020).

Concerns in recent years regarding the expansion of filarial nematodes in the northern areas, from the endemic areas of Europe (southern, central and eastern) are reinforced by the multitude of studies conducted on the prevalence of heartworm disease cases in domestic and wild animals (Sassnau et al., 2014).

Heartworm disease produced by the species *Dirofilaria immitis* is considered a fatal pathology, due to its pulmonary and cardiac location (Genchi et al., 2007; Ciucă et al., 2020a). Clinical manifestations occur due to the large number of adult nematodes, which causes antigenic reactions and mechanical irritation, clinically expressed by a chronic pulmonary hypertension (Beugnet et al., 2018; Burton et al., 2020). In the literature, the clinical stage of cardiopulmonary dirofilariosis is represented as follows: 1) asymptomatic or with very mild manifestations, such as apathy, dyspnea following physical effort and cough; 2) anemia, dyspnea, progressive weakness, chronic cough, vomiting; 3) syncope, increased heart and respiratory rate (Sonneberger et al., 2020).

The second species, *Dirofilaria repenes* causes an asymptomatic and non-pathogenic infection in the subcutaneous tissue in pets, but in humans this is considered the main species involved in the production of the disease (Otranto et al., 2013; Capelli et al., 2018). The life cycle of these two species consists of five larval stages that develop in both hosts, and of the adult forms which have different locations depending on the species (*D. immitis* – right heart and pulmonary arteries; *D. repenes* – subcutaneous tissue) (Genchi et al., 2009).

The female mosquitoes become infected with microfilariae when feeding on the blood of infected definitive hosts, after which they transform into stage 3 larvae. These are considered the infective forms for the subsequent hosts of the mosquitoes (Hess et al., 2023).

The definitive hosts are represented by more than 30 species of domestic (dogs, cats) and wild animals (foxes, ferrets, coyotes, wolves, badgers, etc.) (Adebayo et al., 2020; Ionică et al., 2022).

Recent studies carried out in Romania have highlighted the presence of both species of heartworm in dogs, cats or wild animals (Hamel et al., 2012; Girdan et al., 2015; Ionică et al., 2015; Ciucă et al., 2016a; Ciucă et al. al., 2016b; Ionică et

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al., 2016; Ionică et al., 2017a; Ionică et al., 2017b; Mircean et al., 2017; Ciucă et al., 2020b; Cîmpan et al., 2022; Ionică et al., 2022).

The *Wolbachia* endosymbiont is found in almost all filaria, having the role of helping the nematodes with their long survival, development and fertility (Ciucă et al., 2018).

The aim of this study was to obtain a current prevalence of canine heartworm disease in Galați county, Romania, comparing current data with studies carried out in previous years, from the same area.

MATERIAL AND METHOD

This study included 40 dogs from a shelter in Galați, Romania. Data on age, gender, hair coat length and medical history were recorded for further processing. Their age range was between 1 and 16 years, with varied hair coat length, and according to medical records, none of the dogs received antiparasitic treatment. Based on the age, the dogs were grouped as follows: young (1-3 years); adults (4-7 years); seniors (8-12 years); and geriatrics (13-18 years). Therefore, 40 blood samples were collected in tubes with anticoagulant (ethylenediaminetetraacetic acid- EDTA) and 40 samples in tubes without anticoagulant. The samples were transported at temperatures of 4ºC, and analyzed in the Clinical Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine in Iasi.

All samples used in this study were collected under standard protocols for management of dogs participating in animal shelters and by the Institutional Animal Care and Use committees of the Faculty of Veterinary Medicine Iasi (Romania).

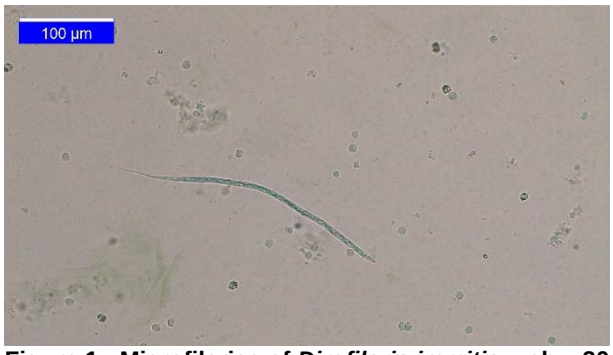

Figure 1 - Microfilariae of *Dirofilaria immitis* – ob. x20

Figure 2 - Microfilariae of *Dirofilaria repens* – ob. x20

Laboratory techniques

Blood samples were tested for the identification of circulating microfilariae using the modified Knott method, as well as the detection of antigens, using the PetCheck and SNAP 4DX Plus serological tests. For the serological tests, canine blood samples were collected in anticoagulant-free tubes, which were after centrifuged, and the serum obtained was transferred to Eppendorf tubes and labeled until use.

Modified Knott test

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 gentle mixing, from the resulting sediment, 1 drop was transferred to a coverslip and examined under an optical microscope (x20-x40) (Gioia et al., 2010; Magnis et al., 2013; Ciucă et al., 2016b). Circulating microfilariae were morphologically identified using specialized

literature (Magnis et al., 2013). the whole sediment was analyzed.

SNAP 4DX Plus immunochromatographic rapid test (Idexx Laboratories)

The first serological test used was the commercial SNAP 4DX Plus kit (Idexx). This qualitative method is used for the detection of circulating *Dirofilaria immitis* antigens, but also for the *Anaplasma platys*, *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Ehrlichia ewingii* and *Borrelia burgdorferi* antibodies detection. Testing was performed according to the manufacturer's instructions. The purpose of using this kit was to perform a more complex screening in order to identify possible co-infections.

PetCheck Heartworm Testing (Idexx Laboratories)

The second serological test used was the PetCheck HW kit (Idexx). This test detects *Dirofilaria immitis* antigens in canine serum or plasma, using the ELISA technique. Testing was performed according to the manufacturer's instructions.

The results of the both serological tests were scored as positive or negative.

RESULTS

Of the 40 dogs, all were homeless (n=40), and regarding the gender ratio, 62.5% were female (25/40) and 37.5% were male (15/40).

The 40 blood and serum samples were tested using the methods described previously. Thus, the modified Knott test showed a total of 11/40 samples positive for microfilariae (27.5%), of which 10/11 samples were positive for *D. immitis* microfilariae (*figure 1*) and 1/11 for *D. repens* microfilariae (*figure 2*). Also, *D. immitis* antigens were detected in 12/40 samples using the PetCheck ELISA (30%), and no samples were positive for SNAP 4DX Plus (*table 1*).

The highest prevalence was detected in the age group 4-7 years (adults), followed equally by the groups 8-12 (senior) years, respectively 13-18 years (geriatrics). In addition, in the gender ratio, 33.4% (25/40) were female and 37.5% (15/40) were male (*table 2*).

The results of the positive samples, tested by the PetCheck HW and modified Knott methods are reported in *Table 3*. Thus, of the 12 samples positive for *D. immitis* Ag. (in Petcheck HW), in 3 of them the microfilariae of both species were identified by the Knott test (*Dirofilaria immitis* and *Dirofilaria repens*), in 5 samples only the microfilariae of the species *D. immitis* were identified, and in 4 samples no microfilariae were identified. Furthemore, from the samples found negative for *D. immitis* Ag., the results from modified Knott method proved that 2 more blood samples were positive for *D. immitis* microfilariae and one for *D. repens* microfilariae.

Table 1 – The results of analyzed samples

Test	Positive samples	
	Total positive/total sampled	**%**
PetCheck HW	12/40	30%
Knott Test	11/40	27.5%
SNAP 4DX Plus	0/40	0%

Table 2 - Comparison of age groups and gender in dogs tested positive for *Dirofilaria* spp. by at least one method

AGE	**No. of dogs tested**	**No. of dogs positive**
1-3 years	8	0 (0%)
4-7 years	14	7 (50%)
8-12 years	10	4 (40%)
13-18 years	8	4 (50%)
Total	**40**	**15 (37.5%)**
GENDER		
Males	15	5 (33.4%)
Females	25	10 (40%)
Total	**40**	**15 (37.5%)**

Table 3 - Comparative results of positive samples

	PetCheck HW	Knott test		Total no. of canine samples
		Dirofilaria immitis	*Dirofilaria repens*	
Sample 8	positive	negative		40
Sample 11	positive	mf	mf	
Sample 12	positive	mf	mf	
Sample 14	positive	mf	mf	
Sample 21	positive	mf	-	
Sample 22	positive	negative		
Sample 24	positive	negative		
Sample 27	positive	mf	-	
Sample 28	negative	mf	-	
Sample 29	negative	mf	-	
Sample 30	positive	mf	-	
Sample 31	positive	mf	-	
Sample 33	positive	negative		
Sample 35	negative	-	mf	
Sample 40	positive	mf	-	

Note: mf - microfilariae

DISCUSSIONS

In this study, are presented the results obtained from the examination of blood samples of 40 dogs randomly chosen from Galați using parasitological and serological methods, in order to establish the prevalence of canine heartworm in the county.

Following studies carried out in previous years, it was found that the highest prevalence of canine heartworm disease is found on the South-East of Romania (Poliana et al., 2013; Ionică et al., 2015; Ciuca et al., 2016; Cimpan et al., 2022). Galați County is located in the South-East of Romania, near the Danube river and other important rivers, thus generating an optimal habitat for the development of vectors. In a research study conducted at the Danube Delta Reservation, certain mosquitoes and domestic dogs were found to exhibit a higher prevalence of *Dirofilaria immitis* and *Dirofilaria repens* infections. (Tomazatos et al., 2018). The study carried by Ciucă et al. in 2016 showed a 60% prevalence of heartworm disease in Galati, the highest rate from all the investigated areas from Eastern Romania.

In the literature, there are described several risk factors involved in the persistance of the disease, including dog gender (males are more susceptible than females in contracting the disease), size (larger dogs are more prone than small ones), habitat, definitive host species, as well as age (older dogs are more susceptible than young ones) (Fan et al., 2001). Comparing these factors with the data presented in this study, it is founded that the prevalence of heartworm disease was increased in females (10 positive samples/25 analyzed samples), and adult dogs, aged between 4-7 years.

The analysis of the 40 blood samples by the Knott method revealed that 11/40 samples were positive for microfilariae, of which co-infections with both species (*D.immitis* and *D.repens*) were detected in 3 blood samples. This fact was also reported by Ionică et al. in 2017 (2017a). They considered that, in endemic areas, the risk of co-infections with both species is very high, especially because these parasites are having common vectors for transmission. In addition, 2 samples positive for *D. immitis* microfilariae were negative on serological testing, most likely due to a low number of females (Rojas et al., 2015). *Dirofilaria immitis* microfilariae can persist for over 2 years in the bloodstream, even after the death of the adults (Ionică et al., 2015).

From the total of 40 serum samples analyzed, 4 samples were positive for *Dirofilaria immitis* Ag., but they were negative in the modified Knott test. According to literature in the field, this situation can be encountered in cases of subclinical infection or during the incubation period, as well as if adult sterility has been previously induced by some drugs used (Ionică et al., 2015). Serological testing by antigen identification is frequently used for the detection of asymptomatic (occult) infections, but the disadvantage of this method consists in false positive results, due to cross-reactions with other filariae (Laidoudi et al., 2019). False negative serological results could occur in cases where the number of female nematodes is low (Schnyder and Deplazes, 2012; Ionica et al., 2015).

According to the manufacturer's specifications, the sensitivity and specificity of the SNAP 4DX Plus and PetCheck HW tests, which detect the antigen of the species *Dirofilaria immitis*, are identical, more precisely 98% sensitivity, respectively 100% specificity (IDEXX Laboratories). In the study conducted by Courtney and Zeng (2001), the PetCheck test was considered the most sensitive compared to the other serological tests used in the study. However, in our study 12/40 serum samples were positive in the PetCheck test and negative in the SNAP 4DX Plus test. The authors of other studies in the branch consider that if the rapid test SNAP 4DX Plus for the detection of *D. immitis* Ag. is elected, it is necessary to use a second serological test, to increase the predictive value (Pantchev et al., 2009).

In Europe, the presence of several filariae in the blood of dogs has been reported, and filariae of the genus *Acanthocheilonema* are also frequently reported. Differentiation of microfilariae is carried out by the modified Knott method, analyzing the morphological characteristics as well as their sizes. However, sometimes there can be uncertainties regarding the species of microfilariae, and the use of other diagnostic methods (serological or molecular) is also recommended (Little et al., 2018; Laidoudi et al., 2020).

Due to the subclinical form of heartworm disease produced by the species *D. repens*, dogs infected with this species remain undiagnosed, indirectly participating in the maintenance of the pathogen (Capelli et al., 2018).

The accuracy of diagnostic methods is extremely important for veterinarians, thus being able to determine the most effective plan for each patient (Panarese et al., 2020). The main reservoir of *Dirofilaria immitis* and *Dirofilaria repens* is considered to be the dog, although heartworm disease can still be prevented by using macrocyclic lactones (Sassnau et al., 2014).

Global warming is thought to be the main cause of the spread of heartworm disease, closely followed by the transport of untested dogs from endemic to non-endemic areas (Genchi et Kramer, 2020).

Dogs in shelters should undergo periodic prophylactic treatments due to prolonged exposure to tick and mosquito infestations, due to the fact that most animal shelters are built on the outskirts of towns. Animal welfare organizations should also be regularly trained on the treatment and testing of dogs for vector-borne diseases, especially those to be imported from endemic areas.

CONCLUSIONS

The high prevalence of heartworm disease in the South-East of Romania should raise an alarm, making the monitoring and prophylactic treatment of dogs mandatory. Also, heartworm disease should be included in the differential diagnosis of veterinarian clinicians, which need to properly inform the owners about the risks associated with canine dirofilariosis. Taking into consideration the evolution of dirofilariosis cases in dog shelters from Romania in the last years, it should be possible that Global Warming influences the increase in the case numbers of this disease, in accordance with abundance of etiological agents.

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