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## HYGIENE PROCEDURES BEFORE, DURING AND AFTER COW MILKING

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### Abstract

In this review paper, hygienic procedures before, during and after cow milking are discussed. The procedures for maintaining hygienic udders before and after milking, as well as maintaining the hygiene of milking units are described. The description includes the maintenance of milker's hand hygiene, udder disinfection and udder disinfection after milking. It is pointed out that these milking procedures should be carried out in a clean environment and without any disturbance to cows. In addition, the need to control the implementation of these hygiene measures is emphasized. A proper way of carrying out the hygienic procedures essentially contributes to reduction of the number of microorganisms and the number of somatic cells in milk, the frequency of occurrence of mastitis, which significantly affects quality of milk and health of milking cows.

**Key words:** dairy cows, milking hygiene, premilking procedures, post-milking procedures

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## HIGIJENSKI POSTUPCI PRE, TOKOM I POSLE MUŽE KRAVA

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### Kratak sadržaj

U ovom preglednom radu razmatraju se higijenski postupci pre, za vreme i posle muže krava. Opisani su postupci pre i posle muže, kao i čistoća opreme za mužu. U opis je uključena higijena ruku mužača, dezinfekcija sisa pre muže, suvo pranje, uklanjanje nečistoće, čišćenje ili sušenje sisa, kao i postupci dezinfekcije posle muže. Ističe se da se ovi postupci kod muže treba da odvijaju u čistom okruženju i bez uznemiravanja krava. Pored toga, navodi se i potreba kontrole sprovođenja tih higijenskih postupaka. Pravilan način sprovođenja ovih higijenskih procedura suštinski doprinosi smanjenju broja mikroorganizama i broja somatskih ćelija u mleku, učestalosti pojave mastitisa, što značajno utiče na kvalitet mleka, kao i opšte zdravstveno stanje krava muzara.

**Ključne reči:** muzne krave, higijena muže, postupci pre muže, postupci posle muže

### INTRODUCTION

Hygienic procedures before, during and after milking of cows are of great importance for milk hygiene and quality. Cow milking hygiene includes the procedures for maintaining udder hygiene before and after milking, as well as maintaining the hygiene of milking units. The procedures for maintaining cow udder hygiene before milking include the following: proper hygiene of milker's hands, udder disinfection before milking, dry washing, pre-milking test, washing and wiping the teats, especially the tips of the teats. These milking procedures should be carried out in a clean parlour or a barn and without any disturbance to cows. The proper manner of carrying out these procedures



essentially contributes to the reduction of the number of microorganisms, number of somatic cells in milk and the frequency of occurrence of mastitis, which significantly affects the quality of cow milk. The paper aims to look into the most important hygiene procedures before, during and after cow milking.

## **PREMILKING HYGIENE PROCEDURES**

Knowledge and practices of hygienic milk production among dairy farmers are very important for hygiene and quality of milk (Ahmed et al., 2020). Premilking hygienic procedures must be carried out regularly and properly. Detailed reviews of failures in carrying out hygienic procedures during the milking of cows are given in the paper by Hristov et al. (2017). One of the most important procedures is the hygiene of the milker's hands. Cow milkers should wear appropriate gloves, which can significantly contribute to preventing the transmission of the causative agents of mastitis from one cow to another. These gloves should be washed and disinfected properly when they become soiled with milk or any other organic substances. Also, care should be taken to ensure that torn gloves are immediately removed and properly replaced (Mihajlović et al., 2022).

In the management and prevention of mastitis, a multifactorial approach with the focus on milking should be taken into account (Hristov and Relić, 2003; De Vliegher et al., 2018). Of great importance is the impact of applying hygienic practices at a farm on the bacteriological quality and somatic cell count of raw milk (Hristov et al., 2002; Riekerink et al., 2012; Williamson and Lacy-Hulbert, 2013; Pandey et al., 2014, Jónás et al., 2018, Mihajlović et al., 2022). Milkers should familiarize themselves with the most important aspects in the application of cow udder disinfection (Hristov and Stanković, 2002), the disinfection of udders and milking equipment (Hristov et al., 1995), as well as the disinfection of cow udders before and after milking (Hristov et al., 1997). It is very important to carry out a regular assessment of cow, udder, and teat hygiene (Reneau et al., 2005; Cook and Reinemann, 2007; Hristov et al., 2017). Also, it should be borne in mind that the premilking test is an essential procedure that detects abnormalities in milk, and the first signs of clinical mastitis, removes the contaminated residual milk from the teat canal, and activates the oxytocin reflex thereby achieving a good milk flow during milking (Hristov et al., 2017; Mihajlović et al., 2022).

The effect of premilking teat foam disinfection on the prevention of new mastitis rates in early lactation is discussed in detail by Fitzpatrick et al. (2021). The effect of premilking teat sanitation on milk composition, somatic cell count and test-day milk yield in Holstein cows was studied by Jónás et al. (2018).

A review of test protocols for the evaluation of teat disinfectants is presented in the paper by Fitzpatrick et al. (2021). Milkers should disinfect the udder teats before milking by immersing them in an approved disinfectant in order to reduce bacterial counts on the teat skin (Hristov et al., 2002; Gleeson et al., 2009; Baumberger et al., 2016; Fitzpatrick et al., 2018; Gleeson et al., 2018; Fitzpatrick et al., 2021). Nowadays, numerous preparations (iodine, chlorine, chlorhexidine – based and others) are available for disinfecting udder teats before cow milking. Disinfectant before milking should remain in contact with the skin of the teat for 30 seconds in order to enable effective disinfection. In the literature, the use of teat immersion in a disinfectant solution is recommended in relation to the application of disinfectant by spraying because it has been established that spraying the teats does not always result in equal coverage of the teats with the disinfectant (Pankey, 1989; Relić et al., 2006). Commercial preparations available for the disinfection of teats before milking have high free iodine content (2 to 3 ppm). They destroy 99.99% of bacteria on the surface of the skin of the teats when applied for 15 to 30 seconds. They are stable at high pH values and can be used without addition of emollients. The addition of emollients reduces their bactericidal power and thus the effectiveness of submerging teats (Gleeson et al., 2009; Gleeson et al., 2018). Various interferences during spraying can prevent the complete wetting of teats, the most common of which are partially blocked sprayer openings (Hristov and Stanković, 2002; Relić et al., 2006; Hristov et al., 2017). In recent times, automatic teat disinfection systems are installed at the exit of the milking parlour of modern dairy farms. These systems are constantly being improved, but so far they have not been nearly as effective as the immersion method (Paliy et al., 2021).

The effect of teat disinfection before milking on the number of microorganisms on the udder teats and the rates of new mammary gland infections were discussed by Gleeson et al. in 2009 and in 2018. Washing the udder and premilking is very important for the hygienic quality of the milk. The occurrence of new infections of the quarters is significantly reduced if, immediately before washing the udders, a few jets are milked in front of the milker, as this eliminates any microorganisms that might be found in the udder (Pandey et al., 2014). Although a much more limited effect was found compared to the effect of disinfection after milking, disinfection of teats before milking is important, as already emphasized, for reducing the occurrence of environmental mastitis (Pankey, 1989; Relić et al., 2006). Studies of the impact of teat sanitation before milking on milk composition, number of somatic cells and milk yield on the day of testing in Holstein cows were also carried out (Jónás et al., 2018). Of special importance is the routine control of disinfection of cow udders and milking equipment (Hristov et al., 1995; Reneau, 1997; Hristov et al., 2003).

Disinfection before milking should be performed after pre-milking test and wiping the teats or washing and wiping them. The minimum contact time should be 30 seconds. This time is necessary for thorough soaking of the teats with disinfectant and their complete penetration, which is also a very effective way to remove dirt and debris from their skin, through washing and wiping. Like with teat disinfection after milking, contamination of the applicator with faeces should be prevented. The speed of disinfection of teats before and after milking is significant (Fitzpatrick et al., 2021).

## POSTMILKING HYGIENE PROCEDURES

The most important aspects of the application of cow udder disinfection and milking apparatus were described in detail by Hristov and Stanković (2002) and Relić et al. (2006), disinfection of udders and milking apparatus by Hristov et al. (1995), and disinfection of cow udders before and after milking by Hristov et al. (1997). Disinfection of teats after removal of milking units is used in order to prevent new udder infections caused by contagious microorganisms. Disinfectants after milking contain high concentrations of active substances and emollients that prevent drying and cracking of teats. Higher concentrations of the active substance of the disinfectant also contribute to prevention of the occurrence of environmental mastitis, since the teat duct remains open for the next 60 minutes or longer after cow milking. Disinfectant after milking should be applied in such a way as to cover the entire teat including the tip of the teat (Hristov and Stanković, 2002; Relić et al., 2006; Williamson and Lacy-Hulbert, 2013).

Williamson and Lacy-Hulbert (2013) looked into the effect of teat disinfection after milking or before and after milking on intramammary infection and somatic cell count. The quality of milk and occurrence of mastitis largely depend on the level of udder hygiene. Excellent results in the prevention of new infections are achieved by immersing teats in disinfectant solutions, which is one of the basic measures in modern mastitis control and suppression programs, especially those caused by staphylococci and streptococci (Hristov and Relić, 2003; Hristov et al., 1997; Relić et al., 2006). The growing importance of Coliform microorganisms in causing mastitis creates a need to find new disinfectants (Hristov et al. 1995). Basically, care must be taken that the disinfectant has a suitable chemical composition so that it does not irritate the skin and that it is prepared properly, i.e., it must not be too diluted, but it should not remain too concentrated either.

Disinfection of teats after milking removes bacteria that are transferred during the milking process and therefore represents one of the most important measures for the prevention of contagious mastitis (Hristov et al., 1997; Relić et al., 2006). This disinfection should be applied immediately after removing the suction cups because then the teat canal is still open. Applied disinfection, immediately after cow milking, allows the disinfectant to penetrate through the external orifice of the teat canal and establish contact with bacteria that have already entered the teat canal. Thus, if a suitable disinfectant is applied, the destruction of pathogenic bacteria is ensured. Postponing the application of disinfection after removing the teat cups allows microorganisms, such as *Corynebacterium bovis*, to reproduce in the teat canal, which leads to an increase in the number of somatic cells in the milk (Hristov et al., 2002; Hristov and Stanković, 2002; Williamson and Lacy-Hulbert, 2013). Macro and micro lesions on the skin of the teats, which are infected with bacteria, heal slowly. Disinfection of the teats removes bacteria from the surface of these lesions and thus speeds up their healing process. Uneven or cracked teat skin can also be a reservoir for pathogens causing mastitis, such as *Staphylococcus aureus* and *Streptococcus dysgalactiae*. Because of all this, disinfection must cover the teats as a whole (Zhang et al., 2021). There are relatively few sebaceous glands on the skin of the teats. Frequent washing and exposure of wet teats to cold and wind can remove protective fatty acids and lead to cracking of the skin. For these reasons, emollients, most commonly lanolin and glycerine, are added to disinfectant preparations in an amount of up to 10%. When disinfectants are used, emollients should be added immediately before each milking (Zigo et al., 2021). Most pathogenic bacteria from the environment (the causative agents of environmental mastitis) reach the teat tips between milkings and move through the teat canal during milking. Since post-milking disinfectants have a relatively short persistence period (1-2 hours after application), they have a limited effect on the causative agents of mastitis from cows' environment.

Numerous disinfectants for immersing teats are used in practice (iodine, chlorine dioxide, hydrogen peroxide, chlorhexidine, sodium hypochlorite, quaternary ammonia, etc.). It is stated in the literature that the most popular application of iodine in a concentration of 1% is by dipping the teats after milking. Iodine preparations have well-expressed disinfection properties because they are created in the form of iodophors. In addition to iodine, iodophors contain some stabilizing agents that prevent iodine molecules from binding to each other so that they remain free and effectively participate in the disinfection of teat skin (Hristov et al., 1995; Hristov et al., 1997; Reneau, 1997; Hristov and Stanković, 2002; Relić et al., 2006; Zhang et al., 2021).

Maintaining optimal mammary gland health and prevention of mastitis is very important for profitable production in dairy cattle (Hristov and Relić, 2003; Zigo et al., 2021). In order to protect teats from infection by pathogenic microorganisms for a long period of time, barrier teat dips were designed. With barrier teat dip, the disinfectant is retained for a longer period of time and remains active when the cows return to the stalls after milking (Zhang et al., 2021). Automatic back-flush units are available and may be beneficial in certain dairy herds. The procedure of back-flushing of the milking unit contributes to the reduction of the transmission of mastitis pathogens from one cow to another through the milking unit. Back-flushing the milking unit with 30 to 50 ppm of iodine will reduce but not eliminate mastitis-causing organisms (Riekerink et al., 2012).

The choice of a disinfectant for udder disinfection should be based on the determination of pathogenic microorganisms that cause mastitis on the farm of dairy cows, and on examining the condition of teat skin. In addition, the link between the inadequacy of routine disinfection before and after milking and the properties of the disinfectant used, including its concentration and method of storage should be taken into account. A detailed review of test protocols for the evaluation of teat disinfectants is given by Fitzpatrick et al. (2018). Numerous protocols found on the website of The National Mastitis Council (NMC) can be of invaluable help in evaluating the effectiveness of udder disinfection. It is very useful to select teat dips according to the data found in the NMC bibliography. In addition, dairy farms with a high incidence of contagious mastitis should focus on choosing a disinfectant after cow milking, while dairy farms with environmental mastitis should focus on choosing a disinfectant to apply before milking cows (Schukken et al., 2013).

## CONCLUSION

Based on the presented literature data on hygiene procedures before and after cow milking, the following can be concluded:

- Attitude and knowledge of the milker about pre and post milking hygienic procedures are of critical importance for maintaining optimal mammary gland health and prevention of mastitis.
- Hygienic procedures before and after milking should be carried out regularly, properly and systematically because they affect the hygiene and quality of milk.
- Out of all these procedures, maintaining the hygiene of the milker's hands, dry washing before milking and disinfection of teats after milking are particularly significant.

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## Author's Contribution:

SH and BS made contributions to the idea of the publication, organisation of work and writing the manuscript; LjS, and DOA were involved in the writing of the manuscript, DN reviewed the manuscript; SH and BS gave the final approval of the manuscript to be published.

## Competing interest

The authors declare that they have no competing interests.

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## EPIDEMIOLOGICAL AND CLINICAL IMPORTANCE OF CANINE DIOCTOPHIMOSIS

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### Abstract

Dioctophimosis is an endoparasitism in dogs caused by *Dioctophyma renale*, a nematode with an indirect life cycle and high zoonotic potential. Infection in dogs occurs through ingestion of transitional/paratenic hosts containing infective larvae. The preferred site of parasitism is the right kidney, although the parasite may also be ectopically localized in subcutaneous tissue, internal organs, peritoneum, and mediastinum. Since dogs are usually infected with a small number of parasites, the disease is asymptomatic, making the diagnosis of this disease in clinical veterinary practice rather difficult. In addition, as dogs are usually infected with a small number of parasites, the disease is asymptomatic, which makes the diagnosing of this disease in clinical veterinary practice difficult. The long prepatent period, lasting three to five months, makes it impossible to diagnose the disease in dogs younger than six months. Clinical suspicion of dioctophimosis in dogs is established on the basis of anamnestic/epizootologic data and the clinical picture, while the final diagnosis is made *ante mortem* or *post mortem* on the basis of reliable diagnostic parameters and methods. The most practical application in routine diagnosis of canine dioctophimosis is the detection of *D. renale* eggs in urine sediment. When dioctophimosis is suspected in

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carnivores, the differential diagnosis should exclude urinary capillariosis, feline polycystic nephropathy, leptospirosis, renal fibrosis, and nephritis of various etiology. The outcome of treatment is uncertain and includes surgical removal of the affected kidney or drug therapy with avermectin derivatives. Prophylaxis consists of preventing contact of dogs with potential transitional hosts and controlling the feeding of their heat-untreated meat. Because of the potential risks that *D. renale* may have on the health of dog owners, education on the etiopathogenesis and means of occurrence, maintenance, and spread of this nematode is necessary.

**Key words:** *Diocetophyma renale*, dog, kidneys, clinical significance, public health

## EPIDEMIOLOŠKI I KLINIČKI ZNAČAJ DIOKTOFIMOZE PASA

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### Kratak sadržaj

Dioktofimoza je endoparazitoza pasa koju prouzrokuje *Diocetophyma renale*, nematoda sa indirektnim životnim ciklusom i visokim zoonoznim potencijalom. Infekcija pasa nastaje ingestijom prelaznih/paratenih domaćina koji u sebi sadrže infektivne larve. Predilekciono mesto parazitiranja je desni bubreg, uz mogućnost ektopične lokalizacije parazita u supkutanom tkivu, unutrašnjim organima, peritoneumu i medijastinumu. Pošto su psi uglavnom inficirani malim brojem parazita, oboljenje protiče asimptomatski, što u kliničkoj veterinarskoj praksi otežava dijagnostiku ovog oboljenja. Dug prepatentni period, koji traje od tri do pet meseci, onemogućava dijagnostiku oboljenja kod pasa mlađih od šest meseci. Klinička sumnja na diok-

tofimozu kod pasa postavlja se na osnovu anamnestičkih/ epizootioloških podataka i kliničke slike, dok se precizna dijagnoza utvrđuje ante mortem ili post mortem na osnovu pouzdanih dijagnostičkih parametara i metoda. Najveću praktičnu primenu u rutinskoj dijagnostici dioktofimoze pasa ima nalaz jaja *D. renale* u sedimentu urina. Kod sumnje na dioktofimozu mesojeda diferencijalno dijagnostički treba isključiti urinarnu kapilariozu, policističnu nefropatiju mačaka, leptospirozu, fibrozu bubrega i nefritise različite etiologije. Lečenje je neizvesno i podrazumeva hirurško uklanjanje promenjenog bubrega ili medikamentoznu terapiju derivatima avermektina. Profilaksa je zasnovana na sprečavanju kontakta pasa sa potencijalnim prelaznim domaćinima i kontrolisanje ishrane njihovim termički neobrađenim mesom. Zbog potencijalnih rizika koje *D. renale* može imati po zdravlje vlasnika pasa, neophodna je edukacija o etiopatogenezi i mogućnostima pojave, održavanja i širenja ove nematode.

**Ključne reči:** *Diocotphyoma renale*, pas, bubrezi, klinički značaj, javno zdravlje

## INTRODUCTION

*Diocotphyoma renale* (giant kidney worm) is a widely spread nematode that parasitizes dogs in tropical and subtropical regions and tends to spread to other geographical areas (Taylor et al., 2007; Eiras et al., 2021).

The parasite is predominantly localized in the right kidney of dogs but can also parasitize in subcutaneous tissues, mediastinum, peritoneum, and internal organs when it causes an ectopic form of the disease (Taylor et al., 2007; Angelou et al., 2020; Greer et al., 2021). During the indirect developmental cycle and migration of larval stages, *D. renale* causes nonspecific symptoms in the form of lumbar pain, dysuria, hematuria, and general disturbances (Gherman, 2013; Russo et al., 2022). The above clinical manifestations are the result of high intensity infections where death due to renal failure is possible (Taylor et al., 2007; Paras et al., 2018).

Larval forms of the parasite cause trauma by migrating through individual tissues and organs - stomach, duodenum, liver, abdominal cavity (Ferreira et al., 2010). Adults inflict chemical damage to infected tissues and organs and, by mechanical action, cause compression and atrophy of the renal parenchyma (Russo et al., 2022) and obstruction of the ureter with subsequent hydronephrosis (Angelou et al., 2020).

In Serbia, there are conditions for the occurrence, maintenance, and spread of this helminthiasis in carnivores and humans related to the presence of infected intermediate/paratenic hosts (oligochaetes, frogs, and fish) and susceptible true hosts (martens, ferrets, foxes, cats, and dogs) in the mentioned epizootic area (Ignjatović, 2022).

There are numerous reports from Serbia related to the problem of intestinal, cardiorespiratory, and ocular parasitic infections in domestic carnivorous animals (Gajić et al., 2014; Ilić et al., 2015; Stepanović et al., 2015; Hadži-Milić et al., 2016; Ilić et al., 2017; Ristić et al., 2020; Stepanović et al., 2020; Ilić et al., 2021) and wild carnivores (Ilić et al., 2012; Ilić et al., 2016; Ilić et al., 2016a; Ilić et al., 2020). Apart from the recent reports on the discovery of *Capillaria plica* species in foxes (Aleksić et al., 2020) and urinary capillariasis in dogs (Ilić et al., 2021a), in previous research in Serbia, not enough attention has been devoted to the study of urinary parasitoses in domestic carnivores.

The aim of this paper is to provide new information on the etiopathogenesis of *D. renale* and point out the need to investigate the occurrence of this parasite in wild and domesticated carnivores in Serbia. The proposed studies are of particular importance because *D. renale* may pose a threat to human health. Since diotrophimosis was diagnosed in dogs in Greece in 2019, the exacerbation of the effects of global warming could be an important predisposing factor for the occurrence of this zoonosis in carnivores in Serbia.

## ETIOPATHOGENESIS

*Diectophyma renale* (Goeze, 1782) belongs to the Animalia kingdom, Nematoda phylum, Enoplea class, Dorylaimia subclass, Diectophymatida order, Diectophymatina suborder, Diectophymatoidea superfamily, Diectophymatidae family, Diectophymatinae subfamily, and *Diectophyma* genus (Gherman, 2013).

### ***Morphological characteristics of the causative agent***

Adult forms of the parasites have a cylindrical bright red body covered with a thin cuticle. Sexual dimorphism is clearly prominent. Females are longer (100-103 cm) and thicker (6-12 mm) compared to males, whose length is about 35 cm, and thickness 3-5 mm. At the anterior end of the body there is a hexagonal shaped mouth, surrounded by 12 papillae arranged concentrically in two rows (Figure 1A). The tale of female parasite is narrow and ends bluntly, while vulva has posterior appearance about the end of oesophagus. The tail

of the female parasite is narrow and ends bluntly, while the vulva is directed backwards over the end of the oesophagus.

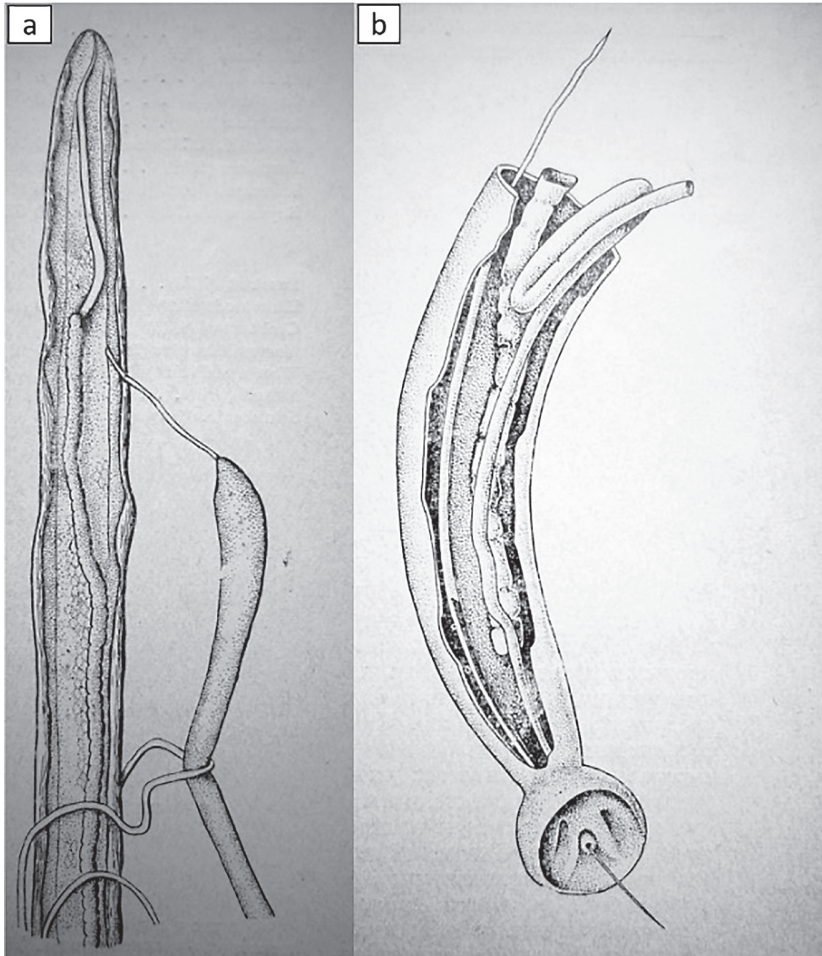


Figure 1. *Dioctophyma renale*: A) anterior side of female body (Goeze, 1782 by Лукасяк, 1930); B) posterior side of male body (Goeze, 1782) (described in Караманова 1968)

At the posterior end of the body of the male worm there is a bell-shaped copulatory bursa with one spicule (Figure 1B and 2). The eggs are oval, yellowish, 74-84  $\mu\text{m}$  in size, unembryonated at the moment of hatching, containing two blastomeres. The egg membrane is thick with one mucoid structure at both poles (Taylor et al., 2007; Gherman, 2013).



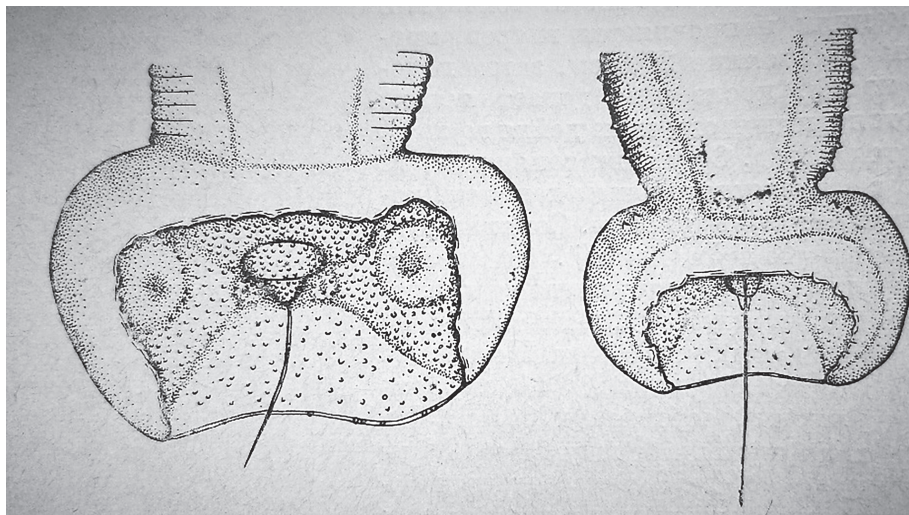


Figure 2. Copulatory bursa of adult male worm *D. renale* (Goeze, 1782 by Стефански, 1928) (described in Караманова 1968)

### ***Life cycle***

Adult forms of *D. renale* are mainly localized in the renal pelvis of the right kidney, while secondary forms can be found in the renal parenchyma, subcutaneous tissue, and abdominal cavity (Gherman, 2013). The prepatent period lasts for approximately 135-155 days, and the complete life cycle is completed in 2 years (Ferreira et al., 2010). Adult female worms hatch eggs, which are ejected- by the urine of infected animals. At an optimal temperature of 25-30°C, first stage larvae (L1) are developed in intermediate hosts for about one month. Low temperatures significantly extend this period (Freitas, 1980).

This parasite has a complex life cycle that includes the presence of intermediate and paratenous hosts. The first intermediate hosts are aquatic oligochaetes (*Lumbricus variegatus*), in which the larvae of the first and second stages are formed (L2). There are three different ways of continuing the transmission. In the first case, infective third (L3) and fourth stage (L4) larvae are formed in the same host. In second, paratenous hosts (fish or frogs) *per os* ingest oligochaetes containing first larval stage forms, which develop in infectious larvae in tissues. A third way of transmission occurs when fish or frogs eat crayfish species from the *Cambarus* genus where infected oligochaetes parasites are present (Angelou et al., 2020).



Dogs become infected by ingesting some intermediate hosts (oligochaetes, fish, or frogs), which contain infective larvae. In the pathogenesis of dioctophimosis, the most important event is the migration of larval forms of parasites, which penetrate the intestinal wall and then reach the abdominal cavity, from where they migrate to the kidneys. The primary place of localization and maturation of the parasite into its adult form is the right renal pelvis, due to its anatomical connection with the duodenum (Ferreira et al., 2010). The adult forms of *D. renale* live for 3 years in a definitive host, while eggs in optimal environmental conditions can survive for 5 years (Angelou et al., 2020).

During the migration through various organs of the infected dog, *D. renale* causes mechanical damage, especially in the right kidney by parenchymal compression. Apart from the mechanical effects, parasites have esophageal glands, whose secretion of lipolytic and proteolytic enzymes causes chemical damage to infected tissues and organs (Russo et al., 2022).

## **GEOGRAPHICAL DISTRIBUTION AND EPIZOOTIOLOGICAL CHARACTERISTICS OF *D. RENALE***

*Dioctophyma renale* is very widespread in tropical and subtropical regions with a tendency to spread to other geographical areas. This phenomenon mainly occurs due to transport of dogs from endemic areas where they get infected by eating meat from intermediate hosts containing infective larvae (Measures, 2001; Russo et al., 2022). It is most commonly diagnosed in North and South America, Asia, and sporadically in Europe (Taylor et al., 2007). According to the literature, *D. renale* has not been diagnosed in Africa and Oceania yet (Acha and Szyfres 1986; Measures, 2001).

The highest prevalence of dioctophimosis is found in mammals from South America (37-42.1%), where the disease has been reported in thirty-three countries with most clinical cases in Argentina (1,414). Due to tropical ecosystems, which are suitable for the coexistence of intermediate and definitive hosts, there is an increasing likelihood of the occurrence, maintenance, and spread of the infection, while humidity and suitable temperature favourably affect the development and vitality of eggs (Russo et al., 2022).

Although the disease in dogs is sporadically present in Europe, the reported prevalence in Poland is 10.5%, indicating a risk of spreading to surrounding countries (Eiras et al., 2021). Dioctophimosis has not yet been diagnosed in dogs from Serbia. Sporadic cases in some countries in Europe, whose number is continually rising, sudden climate changes, increased commercial and un-commercial dog transport worldwide are the factors which contribute to pre-conditions for occurrence of this parasitosis in Serbia as well (Ignjatović, 2022).

Infection in humans by this nematode is described in Australia (Fernando, 1983) and other countries, including Austria, Bulgaria, Chile, Korea (Eiras et al., 2021), Indonesia (Sardjono et al., 2009), Greece (Katafigiotis et al., 2013), India (Venkatrajaiah et al., 2014), Thailand (Beaver and Khamboonruang, 1984), Iran (Norouzi et al., 2017), and Serbia (Nikolić Svetozarević et al., 2001), while cases of *D. renale* infections in mammal animals haven't been reported (Eiras et al., 2021). It is believed that human cases in these countries are of autochthonous origin, unlike recently diagnosed cases of subcutaneous dioctophimosis in Chinese people (one woman and one man) in Japan (Urano et al., 2001; Tokiwa et al., 2014; Tanaka et al., 2020).

The intensity of infection is quite variable and depends on host species and localization of helminths in their organism. High intensity infection considered to be the one with at least twenty adult forms of parasite per one individual (Eiras et al., 2021).

Despite the fact that high temperatures are appropriate for egg development, dioctophimosis is also present in areas with colder weather, which is suitable for intermediate hosts. Other factors like the pH value of an area, salinity of water surfaces, and mobility of intermediate hosts can be responsible for the growing prevalence of dioctophimosis in the mentioned regions (Pedrassani et al., 2009).

Primary environmental contaminants are affected carnivores who eject eggs by the urine. Secondary sources of contamination are intermediate hosts, in which larvae are developed from eggs and continue the life cycle of the parasite (Gherman, 2013).

The factors like ways of keeping and feeding animals, weather disasters (floods) can significantly increase the risk for occurrence of the infection. That is especially true for dogs who live in yards and have close contact with watercourses and are fed raw fish or frogs. Since immunosuppression occurs due to frequentative pregnancies (especially in stray bitches), females are more commonly affected than males. However, males are more territorial with regard to females, and infected prey is more accessible to them. Despite the stated assumptions, gender and age predisposition for dioctophimosis isn't defined in dogs (Pedrassani et al., 2017).

Lack of reports on the prevalence of *D. renale* infections in animals from different regions is a result of insufficient epidemiological studies or the low prevalence and complex life cycle of the parasite (Eiras et al., 2021).

## CLINICAL DIAGNOSTIC PARAMETERS OF DIOCTOPHIMOSIS IN CARNIVORES

Long prepatent period (3-5 months) complicates diagnosis of the disease in dogs under six months of age. Clinical suspicion of dioctophimosis in dogs can be established on the basis of anamnestic or epizootiological data and a clinical picture.

### *Anamnestic and epizootiological data*

During the collection of anamnestic data, it is significant to ask dog owners about feeding habits, contact with water surfaces or aquatic animals (such as frogs or fish), and the possibility of roaming around without owner's supervision (Russo et al., 2022).

The data on lifestyle and quality of life of carnivores collected from owners and available data on climate parameters, transport of dogs and cats in endemic areas of dioctophimosis, or buying pets from risky epizootiological localities, could be very significant for raising suspicion about this nematode (Ignjatović, 2022).

### *Clinical picture*

Nonspecific clinical symptoms of dioctophimosis are not sufficient to make a diagnosis, but they may serve to raise a reasonable suspicion of this disease.

In the clinical picture of dioctophimosis, signs of renal insufficiency are dominant (Gherman, 2013). Symptoms depend on the intensity of the infection and the localization of the causative agent. In low-grade infections, the disease is asymptomatic because one kidney is affected while the other compensates for its impaired function. Then non-specific clinical symptoms like fever, convulsions, abdominal distension, inappetence, and consequently loss of body mass are dominant. In high-grade infections, animals experience haematuria at the end of urination, and pyuria, while the abdomen is painful on palpation in the renal region (Russo et al., 2022).

In the case of an ectopic form of disease, oedema can occur in the inguinal region (it looks like a stab wound with a fistula containing *serohemorrhagic* exudate), proliferation of surrounding tissue (due to chronic peritonitis), and urethral obstruction (the consequences of which are anuria, uremic coma, and death) (Taylor et al., 2007; Paras et al., 2018).

Haematological analysis in the affected dog shows anaemia, eosinophilia, and lymphopenia, while microscopic examination of the blood smear reveals toxic granulation of neutrophils. These findings suggest the presence of acute inflammation. Blood biochemistry results indicate increased blood urea nitrogen and creatinine values. On physicochemical examination, the urine sample is blurred, red, with an alkaline pH value of 8 and contains proteins. After the centrifugation, there is a large amount of urinary sediment (Gherman, 2013; Russo et al., 2022).

A valid *ante mortem* diagnosis is established by parasitological examination of urine sediment, ultrasonography, contrast radiography, computerized tomography, and serological methods of diagnostics (indirect ELISA).

### ***Parasitological diagnostics from urine sediment***

Eggs detection in the urine of an affected dog represents the gold standard in diagnosing this nematode. The urine sample obtained by cystocentesis or catheterisation is centrifuged on 400 spins in 5 minutes with the aim of getting the sediment. After that, the sediment is examined by optical microscope using 40x magnification, and based on morphological characteristics of eggs, *D. renale* can be diagnosed (Figure 1). An advantage of this method is low price, high specificity and sensitivity. However, eggs absence cannot eliminate suspicion of this disease with certainty, especially in cases of ectopic forms, the absence of females, or presence of male adults (Pedrassani et al., 2017). Eiras et al. (2021) report about the rare but possible finding of eggs in animal faeces, which seems to be a result of urine contamination.

### ***Ultrasonography***

Ultrasonography is a fast, effective, and non-invasive technique for diagnosing multifocal circulatory structures that represent longitudinal and transversal sections of a parasite located in the right kidney. Within the ultrasonographic diagnostics it is useful to apply doppler ultrasonography technique that can detect reduction or complete absence of its signal, indicating tissue damage potentially caused by the presence of parasite (Pedrassani et al., 2017; Eiras et al., 2021; Russo et al., 2022).

### ***Native radiography***

Native radiography does not provide valid results, and therefore it is applied in combination with contrast urography, which detects deviations in kid-

ney size, shape, and topography. Based on the elimination of contrast medium, the preservation of kidney function is estimated, which is a significant parameter for the selection of an adequate therapy procedure (Eiras et al., 2021; Russo et al., 2022).

### ***Computerised tomography***

CT scanner is a valid method of diagnostics. Due to its cost, it is primarily used in human medicine, and rarely in affected dogs. Circulatory structures with contrast medium deposits in peripheral parts of tissue are considered positive findings (Eiras et al., 2021).

### ***Serological methods***

In cases of ectopic forms of disease, absence of *D. renale* eggs in urine, or presence of immature females or only males, alternative diagnostic procedures for detecting dirofilariosis include indirect ELISA as well. This test has high specificity of 93 %, it consists of soluble antigens that are obtained by extraction from oesophagus of parasite and it is based on specific antibodies (IgG) detection in blood serum (Pedrassani et al., 2017).

*Post mortem* diagnosis is established on the basis of parasitological sections and histopathology examinations (Ferreira et al., 2010).

### ***Parasitological section***

Since the infections by *D. renale* nematode in cats and dogs are usually low intensity, pathomorphological changes could be less prominent or are completely absent.

In high intensity infections, necropsy has the following findings: thicker kidney capsule, connective tissue infiltration with signs of cystic degeneration. As a result of compressive atrophy on transversal sections, thinned regions of cortex and medulla are common. As a result of urethral obstruction by adult parasite forms, the renal pelvis is highly dilated due to hydronephrosis and filled with haemorrhagic exudate. Due to compensatory hypertrophy and glomerulonephritis, left kidney is bigger compared to the right (Hallberg, 1953; Angelou et al., 2020).

Ectopic migration often occurs when larvae or adult helminth forms are found in the abdominal cavity (Shahbazi et al., 2017), causing peritonitis and perihepatitis (Taylor et al., 2007), or rarely in the thoracic cavity, like perforation

of the mediastinal part of the pleura (Russo et al., 2022). Interference of parasites around hepatic lobes is possibly resulting in capsule erosions, bleeding and rupture (Angelou et al., 2020). In cases of extrarenal localization, larval forms can be encysted in subcutaneous tissue, the spleen, the uterus, the mammary gland, the ovaries, the scrotum, and the testes (Greer et al., 2021), when necropsy is the most appropriate method (Ferreira et al., 2010; Eiras et al., 2021).

In some cases of diotrophimosis, subcutaneous changes are reported. Bitencourt Vidal et al. (2021) report on the accidental finding of nodular structures in the inguinal region of cats, during routine spaying. Parasitological examination of nodular content established the presence of *D. renale*, while histopathological findings confirmed granulomatous inflammation.

### ***Histopathological findings***

Histopathological examination of damaged kidney reveals cortical and medullar atrophy, complete loss of tubular structures, intensive fibrosis and diffuse glomerular sclerosis (Oliveira et al., 2021), neovascularization, hyperaemia, mononuclear cell infiltration (Hallberg, 1953), and dystrophic calcification and metaplastic changes of epithelial cells of the renal pelvis (Russo et al., 2022).

Due to clinical significance of this disease and increasing prevalence during last decade in dogs of surrounding countries, the list of differential diagnosis of diotrophymiasis needs to include the following: nematode *Capillaria plica* (Ilić et al., 2021), chronic nephritis, renal fibrosis and functional kidney disorders of different etiology (Gherman, 2013), as well as polycystic kidney disease of cats and canine leptospirosis (Mesquita et al., 2014).

## **THERAPEUTIC AND PROPHYLACTIC APPROACH**

The most common approach in therapy of diotrophimosis is surgical removal of pathologically changed kidney. In cases of extrarenal localization, laparoscopy is used for larval removal and adult nematode forms. In cases of rare finding of *D. renale* in both kidneys, the only therapeutic method is helminths' extraction from renal pelvises, with uncertain prognosis and survival time from six months to three years (Greer et al., 2021; Eiras et al., 2021).

Medication therapy is extremely limited and uncertain, but avermectine derivatives that can be used are moxidectine and doramectine (Russo et al., 2022). One of effective therapeutic protocols implies intramuscular application of 5 % enrofloxacin (1 ml/10 kg) for five days, then doramectine (1 ml/20 kg) for three days. Seven days later, tablets of enrofloxacin are applied for

three days *per os* and doramectine injection once a week (Eiras et al., 2021).

Greer et al. (2021) designed a protocol that, beside diagnostic methods, also gives information about treatment options for affected dogs according to the results of specialized clinical examination methods (Figure 3). If urinalysis detects *D. renale* eggs, a patient is a candidate for surgical removal of the affected kidney. Before this invasive intervention, it is necessary to examine blood biochemistry parameters that indicate the degree of preservation of kidney function (blood urea nitrogen, creatinine, and electrolytes). If they are within reference values, the patient undergoes surgical intervention and after the surgery is treated with moxidectine (2,5 mg/kg monthly). If kidney function isn't preserved, euthanasia is justified. When there is absence of eggs in urine sediment, ultrasonography is performed. If adult parasite forms are found in the renal pelvis, the previously mentioned procedure is implemented.

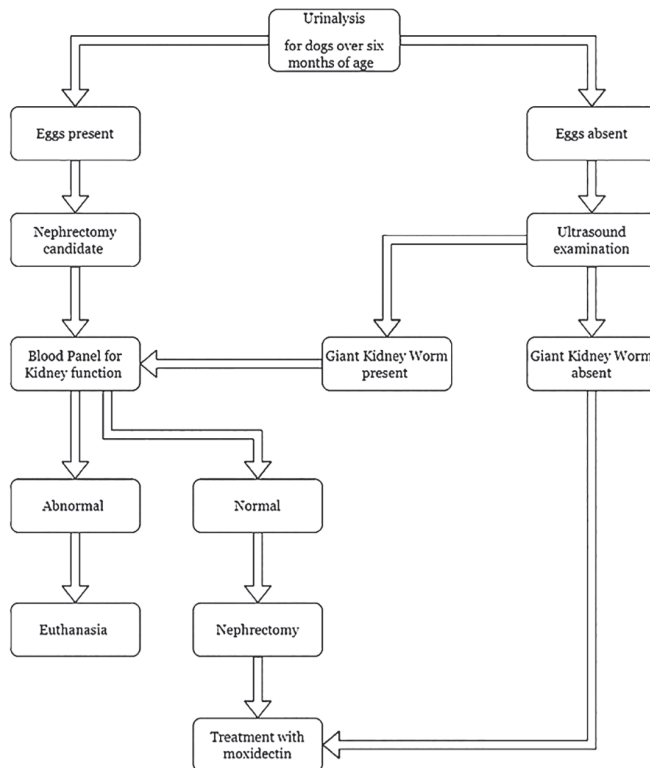


Figure 3. Protocol for diagnosing and treating dogs older than six months infected by *D. renale* (Greer et al., 2021)

Experimental investigations indicate that fungal proteases and chitinases extracted from *Pochonia chlamydisporia* have a harmful effect on the causative agent's eggs. Eiras et al. (2021) suggests the potential possibility of biological control of dioctophimosis and the need for additional investigations.

Control of dioctophimosis is accompanied by the use of general prophylactic measures that are educational for owners because specific measures like vaccination aren't available. Due to the high zoonotic potential of *D. renale* nematode, prevention of infection is especially important for stray dogs because of their uncontrolled movement and contact with ecosystems that represent the source of this parasitosis (Gherman, 2013; Russo et al., 2022). It is necessary to limit the movement of pet dogs or keep them monitored and under supervision in order to avoid contact with water courses, which are habitats for intermediate and paratenic hosts for *D. renale*. It is required to limit the feeding of dogs with raw fish, amphibians, or other intermediate hosts (Gherman, 2013; Russo et al., 2022).

The therapeutic prophylactic approach is very limited by the progressive development of parasitic resistance to some anthelmintic chemical groups.

## **SIGNIFICANCE OF *DIOCTOPHYMA RENALE* NEMATODE FOR PUBLIC HEALTH**

Humans can become infected with the *D. renale* nematode after eating raw fish or frogs containing infective larvae or by ingesting aquatic oligochaetes of the Annelida genus (Chauhan et al., 2016). Due to its nonspecific clinical presentation and rarely diagnosed cases of the disease, this parasite is often neglected in human medicine (Yang et al., 2019). The disease is common in people from Asia (China, Indonesia, Iran, Thailand, and Japan), Europe (Greece and Serbia), North America, and Australia. Most cases are diagnosed in China, which is attributed to their traditional diet and consumption of raw meat (Yang et al., 2019).

Previous studies have found a slightly higher prevalence in males compared to females (59.5% and 40.5%) (Perera et al., 2021). Nematodes can survive in the kidneys for up to five years and cause obstruction, hydronephrosis, and destruction of the renal parenchyma (Chauhan et al., 2016). Affected patients usually have nonspecific symptoms suggestive of nephritis: Haematuria and pain in the lumbar region, while fever, abdominal pain, anaemia, and loss of body mass are less commonly reported. More severe cases are confirmed to be fatal (Perera et al., 2021).



The diagnosis of dioctophimosis in humans is made on the basis of the detection of eggs and adult forms of *D. renale* in a urine, parasitological dissection, and histopathological examination of infected tissue from the right kidney. This finding is explained by the fact that the right kidney is closer to the stomach. According to Yang et al (2019), the parasite presence in the renal pelvis destroys the integrity of the renal tissue and leads to metaplastic changes. This is the reason why the parasite finding is related to the occurrence of renal tumours. The occurrence of ectopic parasitism should not be neglected when *D. renale* larvae are localized in the subcutaneous tissue and retroperitoneal cavity and adult forms are localized in the thoracic cavity, causing inflammatory nodules and skin papules characterized by marked pruritus (Yang et al., 2019). The first diagnosed case of subcutaneous dioctophimosis in a Chinese man who has lived in Japan for 15 years was confirmed by molecular identification of *D. renale* larvae (Tokiwa et al., 2014).

There is still no adequate treatment protocol for human patients, and nephrectomy is considered too invasive. Although there are confirmed successful treatments with ivermectin and albendazole, the use of anthelmintics is still not accepted as a safe therapeutic option. Therefore, in human clinical practice, it is desirable to include this parasitosis in the list of differential diagnoses for patients with nonspecific symptoms such as haematuria and lumbar pain (Yang et al., 2019). The most effective way of prevention is to avoid eating raw fish or frog meat (Chauhan et al., 2016).

Thus far, one case of dioctophimosis has been reported in Serbia in a person who consumed minced fish meat during a trip. Upon his return to Serbia, the patient exhibited the following symptoms: fever, haematuria, dysuria and pain in the lumbar region. The diagnosis was confirmed after surgical intervention and pathohistological examination of the extirpated material (Nikolić Svetozarević et al., 2001).

## CONCLUSION

From a veterinary point of view, dioctophimosis has a high value in clinical practice because dogs are usually affected by only a few parasites. The fact that the disease is often asymptomatic complicates its diagnosis. For this reason, only a reasonable suspicion can be made during the clinical examination of dogs, while it is essential to include the disease in the list of differential diagnoses of urinary tract diseases in carnivores. Because of the high zoonotic potential of the *D. renale* nematode, effective control and management of this helminthosis is crucial. It is also necessary that all fields of human and veterinary medicine work closely together within the concept of "one health".

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## Author's Contributions:

All authors (IT, IN, JMN, DD, NK) contributed to manuscript design, performed literature searches, wrote and revised the article, and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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## EPIDEMIOLOGICAL CHARACTERISTICS OF Q FEVER IN THE SREM DISTRICT OF VOJVODINA, FROM 2011 TO 2020

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### Abstract

Q fever is a globally widespread zoonosis, which has a large number of animal reservoirs. In humans, Q fever is asymptomatic in 60% of cases but may appear in the form of acute or chronic disease. The disease is mostly asymptomatic in animals but may be a cause of abortions or stillbirths. The aim of this study was to present and analyze the epidemiological characteristics of Q fever in Srem district, in the Autonomous Province of Vojvodina, Republic of Serbia. We conducted a descriptive study using data from the registries of infectious diseases of the Institutes of Public Health of Vojvodina and Institute of Public Health Sremska Mitrovica in the period 2011-2020. A total of 76 human cases of Q fever were registered in Srem district over ten-year period. The majority of patients (89.47%; N = 68) were regis-

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tered in municipalities of Sremska Mitrovica and Šid. One third of patients were hospitalized (32.89%; N = 25). Males were 3.2 times more affected than females (M : F = 3.22 : 1). Working age population (20–59 years) were most affected (82.89%; N = 63) with a significantly higher incidence compared to the age category 0–19 years ( $p < 0.05$ ). The majority of cases were registered in February (60.53%; N = 46). Probable routes of transmission of *Coxiella burnetii* were: direct contact with animals and the airborne route of transmission (50.00% vs. 47.37%). The highest seroprevalence of Q fever recorded in goats (55.54%) and sheep (11.88%) in the municipality of Šid, which is connected to outbreak in Kukujevcu 2017. The high seroprevalence in goats, recorded in Indija (22.87%) was not accompanied by Q fever in humans. Q fever still represents a significant problem of both veterinary and human medicine and therefore continuous disease monitoring and more efficient cooperation of the veterinary and human health care sectors is necessary.

**Key words:** Q fever, zoonosis, disease outbreaks, epidemiology, incidence, Srem

## EPIDEMIOLOŠKE KARAKTERISTIKE Q GROZNICE U SREMSKOM OKRUGU, U VOJVODINI, U PERIODU OD 2011-2020. GODINE

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## Kratak sadržaj

Q groznica je globalno rasprostranjena zoonoza, koja ima veliki broj rezervoara među životinjama. Kod ljudi, Q groznica je asimptomatska u 60% slučajeva, ali se može javiti u formi akutne ili hronične bolesti. Bolest je uglavnom asimptomatska kod životinja, ali može biti značajan uzrok pobačaja i mrtvorodenosti. Cilj ovog istraživanja bio je da prikaže i analizira epidemiološke karakteristike Q groznice u Sremskom okrugu, u Autonomnoj Pokrajini Vojvodini, Republika Srbija. Sproveli smo deskriptivnu studiju koristeći podatke iz registara zaraznih bolesti Instituta za javno zdravlje Vojvodine i Zavoda za javno zdravlje Sremska Mitrovica u periodu 2011-2020. godine. Ukupno 76 humanih slučajeva Q groznice je registrovano u Sremskom okrugu tokom desetogodišnjeg perioda. Većina obolelih (89,47%; N = 68) registrovana je na teritoriji opština Sremska Mitrovica i Šid. Trećina pacijenata je bila hospitalizovana (32,89%; N = 25). Muškarci su 3,2 puta češće obolevali u odnosu na žene (M : Ž = 3,22 : 1). Radno sposobno stanovništvo (20-59) je bilo najviše pogođeno (82,89%; N = 63) sa značajno višom incidencijom u poređenju sa starosnom grupom 0-19 godina ( $p < 0,05$ ). Većina slučajeva registrovana je u februaru (60,53%; N = 46). Verovatni putevi prenosa *Coxiella burnetii* su bili: direktni kontakt sa životinjama i aerogeni put prenosa (50,00% prema 47,37%). Najviša seroprevalencija Q groznice zabeležena je kod koza (55,54%) i ovaca (11,88%) u opštini Šid, što je bilo povezano sa epidemijom u Kukujevcima 2017. godine. Visoka seroprevalencija kod koza, zabeležena u Indiji (22,87%), nije bila praćena Q groznicom kod ljudi. Q groznica u Sremskom okrugu i dalje predstavlja značajan problem humane i veterinarske medicine zbog čega je neophodan kontinuirani nadzor i još efektivnija saradnja veterinarskog i sektora zdravstvene zaštite ljudi.

**Ključne reči:** Q groznica, zoonoza, epidemije, epidemiologija, inciden-cija, Srem.

## INTRODUCTION

Q fever is a globally widespread zoonosis caused by the small obligate intracellular gram-negative bacterium *Coxiella burnetii* (*C. burnetii*) (Angelakis and Raoult, 2010). The large number of reservoirs, the possibility of transmission by different routes, the resistance of the microorganism and the small infectious dose place *C. burnetii* in category B on the list of agents suitable

for biological weapons (Parker et al., 2006). Among the many reservoirs, for humans the most important are ruminants: goats, sheep and cattle. Infected animals shed pathogens via body fluids. Q fever is mostly asymptomatic in animals, but it causes abortions and stillbirths (Fournier et al., 1998).

The most common route of transmission of *C. burnetii* to humans is by inhalation of contaminated dust or aerosols from infected feces, urine and birth products (Reimer, 1993; CDC, 2019), thus Q fever is an occupational hazard. Q fever is less commonly transmitted through the consumption of raw milk and unpasteurized dairy products, blood transfusions, from a pregnant woman to her fetus, or through sexual contact (Fournier et al., 1998; Maurin and Raoult, 1999; Milazzo et al., 2001). In humans, Q fever is asymptomatic in 60% of cases and in the rest of symptomatic cases, it may occur in the form of acute or chronic disease (Ristić et al., 2018). Due to asymptomatic course, Q fever is often an unrecognized and under-registered disease.

In aim to better understand the reasons for the endemic maintenance of Q fever, we analyzed the epidemiological characteristics of Q fever in Srem district, in the Autonomous Province of Vojvodina (APV), Republic of Serbia, for ten consecutive years (from 2011 to 2020).

## **MATERIAL AND METHODS**

### ***Study area and data collection***

The study was conducted in Srem district, located in its southwestern part of the APV, with a population of 312,278 inhabitants according to the latest population census (SORS, 2011). The source of data was the database from the registries of infectious diseases of the Institute of Public Health of Vojvodina and the Institute of Public Health of Sremska Mitrovica responsible for the surveillance on communicable diseases. The data of Q fever in humans included individual case reports, epidemiological studies, epidemiological questionnaires, reports on laboratory tests, reports of outbreaks and annual reports for the observed ten-year period (2011-2020). According to the European Center for Disease Prevention and Control (ECDC) case definition, we classified Q fever cases as probable and confirmed cases (ECDC, 2018).

### ***Laboratory confirmation of human Q fever***

Laboratory diagnosis of human Q fever from sera samples was confirmed by the enzyme-linked immunosorbent assay (ELISA) at the Serbian Reference Laboratory for Q fever, the Institute of Public Health of Zrenjanin. A specific

antibody response to *C. burnetti* (IgM and/or IgG to phase II antigen) was used as a laboratory criteria for the confirmation of Q fever. Paired serum samples tested at least two weeks apart were taken for patients whose first serum test results were equivocal or negative.

### ***Veterinary data and laboratory confirmation of animal Q fever***

Data on Q fever laboratory confirmation of animals in Srem district were obtained from the Scientific Institute of Veterinary Medicine, Novi Sad. Samples of goats, sheep and cattle were tested under the auspices of mandatory annual monitoring. By order of the Republic Veterinary Inspection, the Veterinary Scientific Institute, Novi Sad conducted epizootiological investigation in the households with livestock in the case of Q fever in humans. In the case of reports of abortions in domestic animals, serological analysis was performed and in case of positive findings, again, an epizootiological investigation in the household was conducted. The ELISA method was used for the analysis of blood samples (detection of IgM/IgG antibodies against *C. burnetti*) of goats, cattle and sheep (OIE, 2021). Data about results of molecular analysis and data about seroprevalence of Q fever in specific years (2011, 2012 and 2013) are lacking and are not presented.

### ***Data analysis and statistics***

Descriptive method was applied. The data were analyzed chronologically, demographically, and topographically for the observed period. We used the basic statistical indicators, general and specific incidence rates. Statistical data processing included descriptive analysis (mean, minimum and maximum, as well as standard deviation). The Mann-Whitney U test was applied to test the differences of Q fever incidence between two of each age group (0 - 19, 20 - 59,  $\geq 60$  years). For all data analyses the SPSS software, version 21.0 was used and  $p$ -value  $< 0.05$  was considered statistically significant. For mapping, we used the program Quantum GIS (QGIS) version 3.4.

## **RESULTS**

A total of 76 cases of Q fever were registered in the period 2011 - 2020, in Srem district. Of the total number of cases, 92.11% ( $N = 70$ ) were laboratory confirmed and 7.89% ( $N = 6$ ) were classified as probable cases. Males were 3.2 times more affected compared to females ( $M : F = 3.22 : 1$ ). The highest incidence rates of Q fever were reached in 2012 (14.41/100,000;  $N = 45$ ) and in

2017 (7.04/100,000; N = 22) (Figure 1). There were no registered cases of Q fever during the four years (2016, 2018, 2019, and 2020) of this observed period.

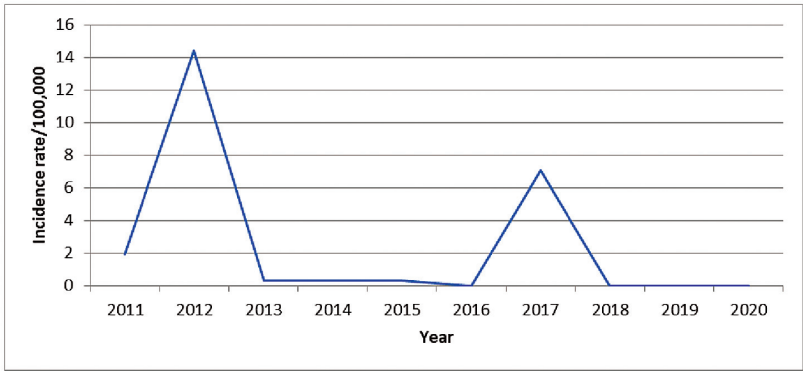


Figure 1. Incidence rate of Q fever in Srem district, in the period 2011 – 2020

Cases of Q fever were registered in all age categories, except in children aged < 10 years. The mean age ( $\pm$  standard deviation) of patients was  $39.51 \pm 14.17$  years. The youngest patient was 14, and the oldest was 75 years old. Age-specific incidence of Q fever during the observed ten-year period is shown in Figure 2. The highest age-specific incidence rates were recorded in 2012, in the age groups 20 - 29, 30 - 39 and 40 - 49 (32.17/100,000; 28.76/100,000; 18.38/100,000 respectively) and in 2017, in the age groups 30-39, 40-49 and 50-59 (16.78/100,000; 16.08/100,000; 7.82/100,000; respectively).

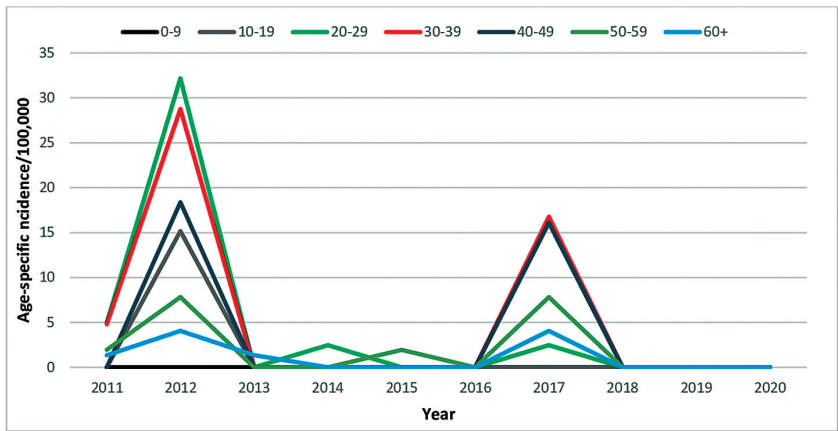


Figure 2. Specific incidence rate of Q fever cases by age in Srem district, in the period 2011 – 2020

The majority of patients (82.89%; N = 63) belonged to the working-age population (aged 20 – 59 years). Age specific incidence (mean, minimum and maximum, as well as standard deviation) are shown in Table 1.

Table 1. Analysis of the age-specific incidence of Q fever in Srem district, in the period 2011 – 2020

Age group (years)	Population <sup>1</sup>	Mean incidence <sup>2</sup>	SD <sup>3</sup>	Minimum incidence	Maximum incidence <sup>2</sup>
0 - 19	61,541	0.76	3.39	0	15.16
20 - 59	176,795	3.66	7.83	0	32.17
≥ 60	73,942	1.08	1.66	0	4.06

<sup>1</sup> according to the latest Census (SORS, 2011)

<sup>2</sup> per 100,000 population

<sup>3</sup> standard deviation

Based on the Q fever data in Srem district during the ten-year period (2011 - 2020), a statistically significantly higher incidence of Q fever was observed in the age category of the working-age population (aged 20 - 59 years) compared to the age category from 0 - 19 years (U = 291.5; p = 0.022) (Table 2).

Table 2. Comparison of Q fever incidence rates by age groups in Srem district, in the period 2011 - 2020

Age group (year)	Mean incidence <sup>1</sup>	Mean Rank	Mann-Whitney U test	p-value
0 - 19	0.76	25.08	291.5	0.022
20 - 59	3.66	33.21		
20 - 59	3.66	25.58	197	0.931
≥ 60	1.08	25.20		
0 - 19	0.76	14.33	86	0.237
≥ 60	1.08	17.85		

<sup>1</sup> per 100,000 population

Regarding the seasonal distribution, the majority of confirmed cases were registered in the period from January to May (96.05%; N = 73). The highest percentage of cases (60.53%; N = 46) was detected in February, and then equally in the months of April and May (13.16%; N = 10) (Figure 3).

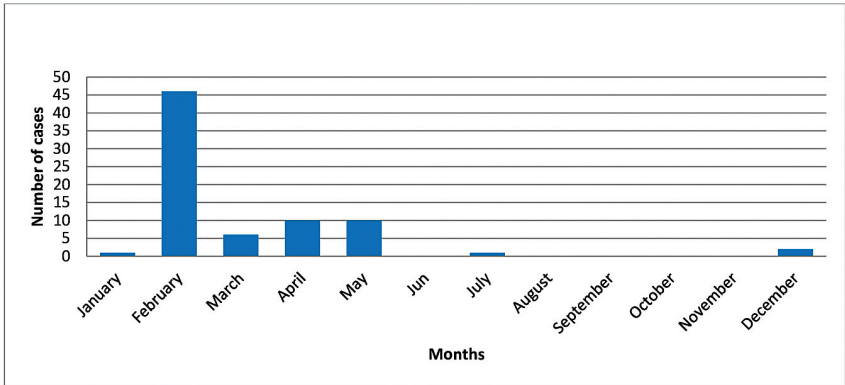


Figure 3. Seasonal distribution of Q fever in Srem district, in the period 2011 – 2020

Most patients (89.47%; N = 68) were registered in the western part of Srem district, in the municipalities of Šid and Sremska Mitrovica (incidence rates 64.35/100,000 and 57.54/100,000; respectively) (Figure 4). The lowest incidence rate (1.52/100,000) was registered in the municipality of Stara Pazova. Cases of Q fever were registered in six out of seven municipalities of the district with the exception of the municipality Indija.

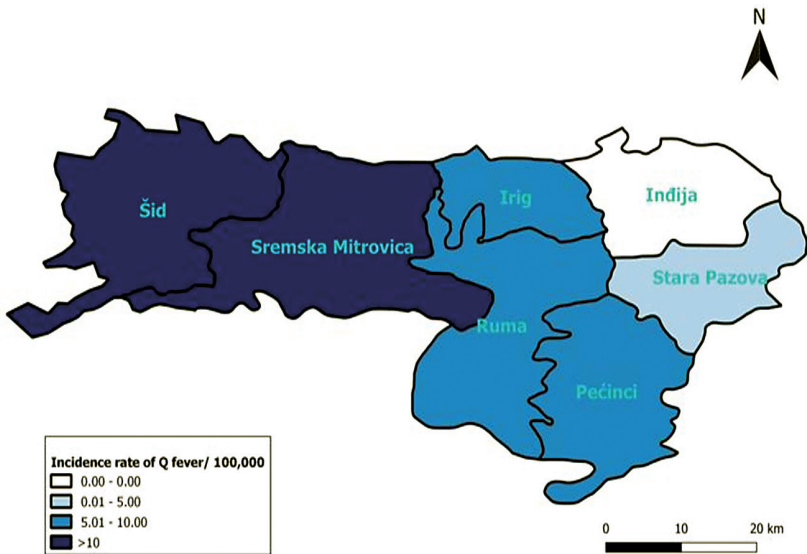


Figure 4. Distribution of Q fever by municipalities in Srem district, in the period 2011-2020

Most (92.11%; N = 70) Q fever cases were detected in outbreaks and the rest (7.89%; N = 6) occurred as sporadic cases. One third of patients (32.89%; N = 25) were hospitalized due to the severity of the disease or complications. There were no registered deaths due to acute Q fever. Direct contact with animals was probably route of Q fever transmission in Srem district, registered in 50.00% (N = 38) of cases (Table 3). Airborne spread of *C. burnetti* was probably route of transmission for the other half of patients (47.37%; N = 36). In only two cases the route of transmission of *C. burnetti* was not established.

Table 3. Possible routes of transmission of Q fever in Srem district, in the period 2011 - 2020

	Direct contact with animals		Aerogenic route of transmission		Unknown route of transmission		Total	
	N <sup>1</sup>	%	N	%	N	%	N	%
Cases in outbreaks	36	51.43	34	48.57	0	0	70	100
Sporadic cases	2	33.33	2	33.33	2	33.33	6	100
Total	38	50.00	36	47.37	2	2.63	76	100

<sup>1</sup> number of Q fever cases

Overall, three outbreaks of Q fever were notified in Srem district in the observed period, in municipalities of Ruma (2011), Sremska Mitrovica (2012) and Šid (2017) with registered 5, 43 and 22 patients, respectively. In the outbreak in the municipality of Ruma, four patients probably contracted Q fever through direct contact with animals (due to occupational exposure), and one patient through airborne transmission. Epizootiological tests were also carried out in six households in the municipality of Ruma (the blood of the sheep and goats was sampled and tested with an ELISA test), and the results for the presence of specific antibodies against the causative agent of Q fever were negative. In the 2017 outbreak, which originated from a goat farm in the village of Kukujevcı (Šid), 17 patients contracted infection during direct contact with animals while working on the farm. The other five patients were residents of the village who probably became infected by airborne route of transmission. Then, in the villages of Kukujevcı and Bačinci (municipality of Šid) and Kuzmin (municipality of Sremska Mitrovica) an epizootiological investigation was carried out, including serosurvey that proved 436 positive animals (149

sheep and 287 goats), of which 409 were seropositive animals in Kukujevcu (278 positive goats on the farm and the rest in the village with the animal keeper) and 27 seropositive animals in one herd in one household in Kuzmin.

During the period from 2014-2020, total of 3,962 animals (821 goats, 3,076 sheep and 65 cattle) were tested for *C. burnetii* in sera samples in Srem district (Table 4). Of all tested animals, the highest seroprevalence was reported in goats (44.58%). The highest seroprevalence was recorded in the municipality of Šid in goats (55.54 %), and also in the municipality of Šid in sheep (11.88%) and in Stara Pazova in cattle (80.00%).

Table 4. Seroprevalence of Q fever in domestic animals in Srem district, by municipalities, in the period 2014-2020

Animal species	Municipalities								Total
		Indija	Irig	Pećinci	Ruma	Šid	Sremska Mitrovica	Stara Pazova	
Goats	T <sup>1</sup>	188	9	20	20	271	2	0	510
	P <sup>2</sup>	43	0	0	0	36	0	0	79
	%	22.87	0.00	0.00	0.00	13.28	0.00	0.00	15.49
Sheep	T	304	162	97	337	831	407	114	2,252
	P	7	6	0	3	61	17	2	96
	%	2.30	3.70	0.00	0.89	7.34	4.17	1.75	4.26
Cattle	T	39	2	11	3	0	5	5	65
	P	0	0	1	0	0	0	4	5
	%	0.00	0.00	9.09	0.00	0.00	0.00	80.00	7.69

<sup>1</sup> tested

<sup>2</sup> positive

## DISCUSSION

Our study shows that Q fever is still endemic disease in Srem district with majority of human cases registered in outbreaks. The highest incidence of Q fever in the observed ten-year period was registered in the western part of the Srem district. Men were more affected as well as the working-age population. One third of patients were hospitalized, none with death outcome. Looking at the season, the largest number of infected people was recorded in February while probable routes of transmission were direct contact with animals and airborne spread of infection.



The results of our study showed that there were more cases among men in Srem district, which may be linked to their higher likelihood of occupational exposure. Due to the protective role of sex hormones, women develop symptomatic Q fever less frequently (Leone et al., 2004). Our findings are similar to the results of several published studies showing that the majority of cases were registered in men (Anderson et al., 2009; Gyuranecz et al., 2014; Halsby et al., 2017; Genova-Kalou et al., 2019).

The average annual incidence rate of Q fever in Srem district in the observed period was 1.7 times higher than the incidence rate notified in APV and five times higher than the average annual incidence rate recorded in the Serbia in the same period (IPHS, 2021). The average annual incidence of Q fever in Srem district (2011 - 2020) was approximately two and 1.2 times lower compared to average incidences registered in the endemic areas of Central Banat and South Banat (IPHS, 2018; IPHS, 2021, Ristić et al., 2018). Compared to the average annual incidence rate of Q fever (incidence of 0.17/100,000) in the countries of the European Union (EU), the average annual incidence rate in Srem district was 14.3 times higher in the period 2011 - 2020 (ECDC, 2017; ECDC, 2021).

The lowest incidence of Q fever in EU, in the observed ten-year period was reported in 2020 (ECDC, 2021), when, interestingly, not a single case of the disease was registered in Srem district, nor in the territory of APV. This is explained by the fact that the Coronavirus disease 2019 (COVID-19) emerged in 2020, which is why all communicable diseases, including Q fever, were under-reported. Besides, it is possible that pandemic mitigation measures such as wearing masks and washing hands also reduced the chance of contracting Q fever, since it is an airborne infection.

In our study, there were no registered cases among children < 10 years which is similar to the situation in EU, where the small number of patients with positive samples was recorded in children under 10 years of age (ECDC, 2017). In accordance with the above, we found a statistically significantly higher incidence of Q fever in the working age population (20 - 59 years) compared to the age category of 0 - 19 years old. The reason for this finding may be that children and adolescents infected with *C. burnetti* are more often asymptomatic than adults and may have milder symptoms of the disease, so cases of Q fever are less often registered among them, which is in agreement with other studies (Maltezos and Raoult, 2002; Terheggen and Leggat, 2007).

Seasonal distribution of Q fever, with the majority of patients in Srem district registered in the period January - May, may be related to the fact that during these months the lambing and kidding of sheep and goats are done and other activities in which humans are in contact with potentially infected

animals. During the huge outbreak in the Netherlands, it was observed that the majority of patients appeared, at the end of winter and at the beginning of spring (Schneeberger et al., 2014). Several studies have pointed out the role of high wind speed and low rainfall on the easier transmission of microorganisms and the higher incidence of Q fever (Tissot-Dupont et al., 2004; Medić et al., 2012; Ristić et al., 2018; De Rooij et al., 2018).

Outbreaks in the village of Noćaj (municipality of Sremska Mitrovica - 2012) and in the village of Kukujevcı (municipality of Šid - 2017) contributed to the peaks of incidence registered in 2012 and 2017 as well as to the highest incidence rates recorded in these municipalities. The reason for this may be the fact that these two municipalities are close to the border of Croatia, where the highest incidence of Q fever in Europe was registered in 2012 and 2017 (ECDC, 2019). Not a single case of Q fever was registered in Indija, even though there was an outbreak in a goat farm with a high number of positive animals. The most possible reason may be the under-registration of human Q fever cases, as well as the asymptomatic clinical course of the disease.

An extensive epidemiological investigation of Q fever outbreak in village Noćaj (Sremska Mitrovica) established that the most likely route of transmission for more than 2/3 of the patients was airborne, while the rest reported direct contact with animals (Medić et al., 2012). Our results also showed that half of patients had no contact with animals and that they most likely contracted Q fever which was transmitted through the air. This claim is in agreement with previous studies that *C. burnetii* can be transmitted by wind through aerosols and dust over long distances (within 5 km of the source of infection) (Arricau-Bouvery and Rodolakis, 2005; Debeljak et al., 2018). Furthermore, the other half of the cases of Q fever were registered in people who had direct contact with animals, similar to other studies conducted in APV (Ristić et al., 2018; Popov et al., 2019).

Some limitations of our study should be listed. First, documentation of Q fever is limited by the availability of laboratory testing, and it is certain that a majority of asymptomatic infections were not recorded. This suggests that the incidence rates of Q fever are likely to be higher than registered. Earlier studies on Q fever found a positive correlation between the number of infected people and animals in Srem district (Ristić et al., 2018), but more extensive studies are needed to address the low incidence or absence of cases in municipalities where positive animals have been registered. No data for the outcomes of chronic forms of Q fever were obtained during this study.

## CONCLUSION

Our results show that Q fever is still an endemic disease in Srem district and represents a significant problem of veterinary and human medicine. More efficient disease control requires strengthening surveillance with continued monitoring the disease in animals, and more effective cooperation between sectors of human and veterinary medicine.

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### Author's Contribution:

SP and SM made contributions to conception and design of the article; SP wrote the manuscript. TP contributed in results analysis and the way of results presenting. ND performed the statistical analysis. SS, DB, ML and JJK were involved in the data collection. SM and TP revised the manuscript critically. SP prepared the final draft of the manuscript. All authors read and approved the final manuscript.

### Competing interest

The authors declare that they have no competing interest.

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## ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM WHEAT, SUNFLOWER AND MAIZE CROP RESIDUES

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### Abstract

Large quantities of agricultural residues are generated every year. Most of the crop-based residues are underutilized, mainly left to decay on the land or to be burnt, which can lead to an increase in a load of environmental pollution. Considering this, different strategies have been developed to use these renewable resources as raw materials for the production of bioactive compounds, their isolation and characterization, and potential application in a wide range of fields, particularly in the food industry as natural preservatives. In this study, the antibacterial efficacy of wheat, sunflower, and maize crop residue ethanolic extracts against six bacterial strains (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica*) was evaluated by the broth microdilution method. Used extracts inhibited the growth of selected microorganisms with a minimal inhibitory concentration (MIC) of 320 µg/mL for most of the tested bacteria. *L. monocytogenes* showed a MIC value of 640 µg/mL for wheat ethanolic extract, and the MIC value

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of sunflower ethanolic extract for *S. Typhimurium* was 160 µg/mL. There were no minimum bactericidal concentration (MBC) values for any of the bacteria within the extract's concentration ranges tested ( $\leq 2560$  µg/mL). The results of the present study indicate that crop residue ethanolic extracts could exhibit bacteriostatic effect and therefore have the potential as natural additives in food preservation.

**Key words:** agricultural waste, MIC and MBC, maize and sunflower stalks, wheat straw, foodborne pathogens

## ANTIBAKTERIJSKA AKTIVNOST ETANOLNIH EKSTRAKATA ŽETVENIH OSTATAKA PŠENICE, SUNCOKRETA I KUKURUZA

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### Kratak sadržaj

Velika količina poljoprivrednih ostataka se proizvodi svake godine. Većina žetvenih ostataka je nedovoljno iskorišćena, uglavnom se ostavlja da propadne na njivama ili se spaljuje, što dovodi do povećanog zagađenja životne sredine. Imajući ovo u vidu, razvijene su različite strategije za iskorišćavanje navedenih obnovljivih resursa kao sirovina u proizvodnji bioaktivnih jedinjenja, njihovu izolaciju i karakterizaciju i potencijalnu primenu u različitim oblastima, naročito u industriji hrane kao prirodni konzervansi. U ovoj studiji ispitivana je antibakterijska efikasnost etanolnih ekstrakata žetvenih ostataka pšenice, suncokreta i kukuruza na šest bakteri-



jskih sojeva (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* i *Yersinia enterocolitica*) primenom mikrodilucione metode u bujonu. Ekstrakti žetvenih ostataka su inhibirali rast odabranih mikroorganizama u minimalnoj inhibitornoj koncentraciji (MIC) od 320 µg/mL za većinu ispitivanih bakterija. Za *L. monocytogenes* MIC vrednost etanolnog ekstrakta pšenice bila je 640 µg/mL, a 160 µg/mL je bila MIC vrednost etanolnog ekstrakta suncokreta za *S. Typhimurium*. Minimalna baktericidna koncentracija (MBC) prema bakterijama nije postignuta u ispitivanim koncentracijama etanolnih ekstrakata ( $\leq 2560$  µg/mL). Rezultati ukazuju da etanolni ekstrakti žetvenih rezidua bi mogli da ostvare antibakterijski efekat i stoga imaju potencijal kao prirodni aditivi u konzervisanju hrane.

**Ključne reči:** poljoprivredni otpad, MIC i MBC, stabljike kukuruza i suncokreta, pšenična slama, patogeni koji se prenose hranom.

## INTRODUCTION

With the growth of the world's population, which in November 2022 reached 8 billion inhabitants, there is a great necessity for the production of a large amount of food (UN, 2022). Besides China and India, which are the two largest agricultural-producing countries, 157 million hectares of land were used for agricultural production in the European Union (EU) in 2020 (Eurostat, 2022). Therefore, modern agriculture produces a considerable amount of residues every year, and its vast majority is currently dumped and accumulated in landfills or burned (Sadh et al., 2018). In Serbia, the total production of three of the most represented crops in 2021 was as follows: maize 6027131 tons, wheat 3442308 tons, and sunflower 607574 tons (Stat.YearB.Serb, 2022). Even though Serbia has a relatively developed agricultural sector (Zekić et al., 2010), agricultural waste is still an underutilized resource (Maksimović, 2022).

There are two types of agricultural residues: field (crop) and process residues. Field residues remain in the field after crop harvesting and consist of leaves, stalks, straws, seed pods, stems, hulls, cobs, and weeds. Process residues are residues present even after the crop is processed into a valuable alternate resource, and these include husks, seeds, roots, bagasse, and molasses (Sadh et al., 2018). The limited and inadequate management of this agricultural waste causing environmental pollution is a global issue that emerged an urgent need to develop strategies based on new sustainable and circular models for waste timely utilization and valorization (Carpena et al., 2022).

Numerous studies have demonstrated that agro-industrial residues are essential sources of various complex and structurally diverse bioactive compounds, including flavonoids, hydroxycinnamic acid derivatives, phenolic acids, tannins ascorbates, lignans, carotenoids, tocopherols, phytosterols and arabinoxylans (Babbar and Oberoi, 2014; Sadh et al., 2018). Hence, mainly process residues are shown to be raw materials with good prospects for extracting and identifying new compounds with antimicrobial and antioxidant potential (Siheem et al., 2015; Sheng et al., 2022). On the other hand, field residues are abundant lignocellulosic biomass that varies slightly in composition with cellulose, hemicellulose and lignin as the major constituents, and the knowledge of their potential as a raw material for the extraction of different bioactive phenolics is limited (Singh and Nigam et al., 2009). Only few data are found in the literature regarding phenolic compounds content in crop field residues, such as wheat, maize and sunflower (Kumar and Goh, 2003; Vijayalakshmi et al., 2015; Alexandrino et al., 2021).

During the last two decades, extensive research has been devoted to discovering new antimicrobial agents, mainly from plants and other natural sources that could be applied in pharmaceutical and cosmetic products, as well as in the food industry (Nazzaro et al., 2013). With the development of the all-natural and green-label trend and consumer awareness of food safety and quality, meat and meat products that are highly susceptible to the growth of spoilage microorganisms and foodborne pathogens are of particular interest in regard to finding natural preservatives that could be used as safe antimicrobials and antioxidants in meat matrix and packaging (Ji et al., 2021).

Various basic standard methods and complex bioassays have been developed for *in vitro* antimicrobial susceptibility testing; the well-known and most commonly used methods include disk diffusion, well diffusion and broth or agar dilution (Balouiri et al., 2016). The microdilution method is considered a valuable tool for detecting the resistance to antimicrobials and comparing different susceptibility since it is a fast method that does not require many resources and provides information on the lowest concentration that inhibits bacterial or fungal growth (Kolarević et al., 2016).

Considering all mentioned above, the present work is designed to investigate the antimicrobial effect of ethanolic extracts from three different agricultural residues - wheat, sunflower, and maize against several microbial strains (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica*) which are the pathogens identified as the most common causes of foodborne diseases.

## MATERIAL AND METHODS

### *Plant materials*

Wheat, maize and sunflower harvest residues (maize and sunflower stalks, as well as wheat straw) originating from the territory of the Autonomous Province of Vojvodina (Serbia) were collected after the harvest time, between July and October 2021 and dried naturally in a shaded and well-ventilated place. A 3 kg quantity of each material was first reduced to smaller particles using a grinder; then extracted with a six-fold weight of hexane for 1 h at 40 °C in industrial stainless steel 60 L extractor. Each of obtained hexane extracts was vacuum filtered through 87 g/m<sup>2</sup> filter paper to remove the hard residues and concentrated using a DLAB RE 200 Pro industrial rotary evaporator (60 °C, 60 rpm, 216-200 mbar, 150 min). After extraction with hexane, plant material was left aside for 24 h in the open air, protected from direct sunlight, in order to remove the traces of residual solvent, and extracted again, for 1 h at 45 °C using a six-fold weight of 96% ethanol, followed by filtration and evaporation under the same working conditions.

Ethanol extracts were used for further investigations. An aliquot of each extract was taken and diluted with DMSO before the analyses, as described further in the text.

### *Test organisms*

Six reference bacterial strains were investigated: *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis ATCC 13076, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19111 and *Yersinia enterocolitica* ATCC 9610.

### *Minimum inhibitory concentration (MIC)*

The susceptibility of the selected isolates to active compounds was investigated by the broth microdilution method (CLSI, 1999; 2020; ISO, 2019). Each crop residue extract was tested in triplicates. The inoculum was prepared by the colony suspension method. The stock control culture of each of the six reference strains was sub-cultured on non-selective nutrient agar (NA) (Oxoid®, UK) at 37 °C for 18 h to 20 h. Three to five pure colonies of each microorganism were touched with a loop and suspended in 5 mL sterile saline. The suspension was adjusted to give a turbidity equivalent to a 0.5 McFarland standard using

a spectrophotometer Cecil 2021 UV/VIS (Select Science, Bath, UK) where at 625 nm wavelength and a 1 cm path cuvette, the absorbance was in the range of 0.08 – 0.13. The prepared adjusted inoculum (approximately  $1 \times 10^8$  CFU/mL) was diluted by transferring 0.1 mL of standardized isolate suspension to a tube containing 9.9 mL of Cation Adjusted Mueller-Hinton broth (CAMHB) (BBL™ Mueller Hinton II Broth, Becton, Dickinson and Company, Sparks, USA) (1:100 dilution) to obtain suspension of  $1 \times 10^6$  CFU/mL, so when 50 µl is added to an equal volume (50 µl) of the examined solution, resulted in a final inoculum of  $5 \times 10^5$  CFU/mL. For *L. monocytogenes* modified susceptibility medium, CAMHB without adding 5% lysed horse blood and 20 mg/mL β-NAD was used (Takahashi et al., 2013). Wheat, sunflower and maize crop residues extracts were diluted in DMSO (Fisher Scientific™, UK) and added to CAMHB at levels from 2560 µg/mL to 1.25 µg/mL by two-fold dilution in U-bottom 96-well microtiter plates (Kartell S.p.A., Italy). After inoculation, plates were incubated at 37 °C for 18-20 h. MIC was determined as the lowest concentration of an active compound that prevented the visible growth of bacteria in the broth dilution susceptibility test (CLSI, 2012). Tetracycline (Fisher Scientific™, UK) was used as a control in the range of 64 to 0.03 µg/mL. The plates also included a negative control (media only) and a bacteria growth control (media and bacteria).

### ***Minimum bactericidal concentration (MBC)***

Following MIC determination of the crop residue extracts and antibiotic yielding a negative microbial growth after incubation, a well's content (10 µL) was sub-cultured on the surface of NA plates to determine the number of surviving cells (CFU/mL). The plates were then incubated overnight at 37 °C. The minimum bactericidal concentration (MBC) endpoint was defined as the lowest concentration of extract that kills > 99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the NA plates (CLSI, 1999). The tests were carried out in triplicate.

## **RESULTS**

Using described methodology, the following yields of ethanol extracts were obtained: sunflower 2.55%, wheat 1.35%, and maize 1.32%.

The *in vitro* antimicrobial activity of ethanolic wheat, sunflower and maize crop extracts and the commercial antimicrobial agent is demonstrated in Table 1. For most of the tested bacteria, the MIC value at the examination of

all three crop residue extracts was 320 µg/mL. The exception was *L. monocytogenes* which revealed MIC value of 640 µg/mL MIC for wheat ethanolic extract, while the MIC of sunflower ethanolic extract for *S. Typhimurium* was 160 µg/mL. There was no MBC value for any of the microorganisms at the extract's concentrations used (> 2560 µg/mL).

Table 1. Minimal inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) breakpoints of examined crop residues extracts for six bacterial isolates

Organism	Wheat		Sunflower		Maize		Tetracycline	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
<i>S. Typhimurium</i>	320.00	>2560.00	160.00	>2560.00	320.00	>2560.00	0.125	64.00
<i>S. Enteritidis</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	1.00	> 64.00
<i>S. aureus</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	1.00	> 64.00
<i>E. coli</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	1.00	> 64.00
<i>L. monocytogenes</i>	640.00	>2560.00	320.00	>2560.00	320.00	>2560.00	2.00	> 64.00
<i>Y. enterocolitica</i>	320.00	>2560.00	320.00	>2560.00	320.00	>2560.00	2.00	> 64.00

## DISCUSSION

The susceptibility of the bacterial strains toward the commercial antibiotic tetracycline used in the present study was in accordance with the data reported in the literature (Musumeci et al., 2003; Purushotham et al., 2010).

The chemical characterization of the main constituents of plant parts that are considered crop residues, including those examined in the present study was summarized by Sadh et al. (2018). Slight differences in the chemical composition among these three crop residues were detected; the highest content of cellulose (61.2%) and the lowest content of lignin (6.9%) were determined in maize stalk residues, sunflower stalks had the highest content of hemicellulose (29.7%), lignin (13.4%) and ash (11.17%), while wheat straw had the lowest content of cellulose (32.9%) and ash (6.7%) (Singh nee' Nigam et al., 2009; Martin et al., 2012; Sadh et al., 2018). Furthermore, previous research

investigated the antioxidant properties of different agro-industrial wastes (Câmara et al., 2020; Carpena et al., 2022); however, the antimicrobial activity has been less studied (Martin et al., 2012; Martillanes et al., 2020; Alexandrino et al., 2021). To our knowledge, there is no data on the antimicrobial effect of ethanolic maize and sunflower stalks, and wheat straw extracts. As can be observed, all three extracts inhibited the growth of gram-negative and gram-positive microorganisms in a similar way, except for the lower sensitivity of *L. monocytogenes* and the highest sensitivity of *S. Typhimurium* to wheat and sunflower extracts, respectively. The determination of the chemical composition of various plant residues revealed the presence of different classes of secondary metabolites with the most abundant phenolic compounds that have been shown to inhibit the growth of foodborne pathogens and spoilage bacteria (Vijayalaxmi et al., 2015; Gomes-Araújo et al., 2021). These phenolic compounds in the wheat, sunflower and maize crop extracts could give a preliminary explanation of the antimicrobial activities observed in the present study. The slight differences in the exhibited activity could be due to the differences in chemical composition, the concentration of the main bioactive constituents, their mechanism of action, and possible interaction with other components in the final extract (Dzotam et al., 2015; Hemeg et al., 2020). Corroborating our results, Alexandrino et al. (2021) demonstrated that the ethanolic extract of defatted sunflower seed flour exhibited antimicrobial activity against *S. aureus*, *Bacillus subtilis*, *E. coli*, and *Pseudomonas aeruginosa* with the MIC values in the range from 11.6 to 33.2 mg chlorogenic acid (CGA) eq/mL. The highest susceptibility to sunflower seed extract showed *E. coli* (Alexandrino et al., 2021), while in our study *S. Typhimurium* was the most sensitive bacteria. The total phenolic content of this sunflower seed flour was 4.00 g CGA eq/100 g on a dry basis. After concentration, the ethanolic extract had a total phenolic value of 15.44 g CGA eq/100 g, with 62% of chlorogenic acid as the predominant phenolic compound (Alexandrino et al., 2021). According to previous reports, among the analyzed phenolic compounds, chlorogenic acid was predominant in defatted sunflower kernels and shells (Weisz et al., 2009). The antibacterial effect of sunflower extracts was mainly attributed to chlorogenic acid as it can bind to the outer bacteria membrane, increase the permeability of the outer and plasma membrane and lead to its damage with the leakage of intracellular components, finally resulting in cell death (Lou et al., 2011). The efficiency of sunflower-based extracts depends on the concentration of chlorogenic acid, that is, on the purity of the obtained extracts after the extraction process and the presence of other compounds in addition to phenolic components, such as soluble sugars or proteins (Alexandrino et al., 2021). Namely, the inhibition of

the growth of various bacterial strains, including *S. aureus*, *Streptococcus pneumoniae*, *B. subtilis*, *E. coli*, *Shigella dysenteriae*, and *P. aeruginosa* is achieved at concentrations from 10 to 30 times lower using chlorogenic acid with  $\geq 98\%$  purity (Lou et al., 2011; Fu et al., 2017).

Martillanes et al. (2020) found that in both aqueous and ethanolic rice bran extract, with trans-ferulic acid, *p*-coumaric acid, and  $\gamma$ -oryzanol as the main components, the growth of *E. coli* and *L. innocua* was inhibited. However, the percentage of inhibition was notably higher in an ethanolic extract with high  $\gamma$ -oryzanol and low phenolic compounds concentration. Contrarily, by examination of methanolic and ethanolic extracts from 20 different agro-industrial wastes, the positive correlation between total phenolic content and antimicrobial activity was confirmed (Martin et al., 2012). Martin et al. (2012) found that, besides the absence of an inhibitory effect against gram-negative bacteria (*S. Enteritidis* and *E. coli*), the best antimicrobial activity against *S. aureus* showed ethanol extract of peanut peel with a MIC value of 0.78 mg/mL and total phenolic value of 374.5 gallic acid equivalent (GAE)/kg, while *L. monocytogenes* growth was inhibited by guava bagasse ethanol extract (1.56 mg/mL) with a total phenolic value of 43.1 GAE/kg. Compared with the results of our study, ethanolic wheat, sunflower and corn crop residues showed higher antimicrobial potency and inhibited both gram-positive and gram-negative bacteria in concentrations twice lower than those obtained by Martin et al. (2012). Among the compounds with antibacterial activity in agro-industrial waste extracts, Martin et al. (2012) confirmed the predominant presence of dicarboxylic acids: azelaic and succinic acids, then caffeic, *p*-coumaric, syringic, gallic, ferulic acids and flavonoids: epicatechin, myricetin, and quercetin. Earlier studies reported 0.9% of polyphenols in wheat crop residues (Kumar and Goh, 2003) and the following organic acids in the wheat straw water extract: *o*-dihydroxybenzene, *p*-hydroxybenzoic acid, ferulic acid, and catechinic acid (Hongzhang and Liying, 2007).

In the present study, 96% ethanol was used for the extraction of polyphenol-rich paste from crop residues, whereas Vijayalaxmi et al. (2015) demonstrated that by using 100% ethanol, the extraction yield was better for wheat bran (3.5%) compared to corn husk (4%) and that the total polyphenols, total tannins and total flavonoids contents in corn husk extract were 35.80 g GAE/100 g extract, 29.33 g tannic acid equivalents (TAE)/100 g extract and 7.35 g quercetin equivalents (QE)/100 g extract, respectively, while in wheat bran these contents were 40.12 g GAE/100 g extract, 33.35 g TAE/100 g and 5.86 g QE/100 g extract, respectively. In that study, HPLC analysis of corn husk and wheat bran extracts detected two major peaks corresponding to gallic acid



and ferulic acid and three minor peaks identified as epicatechin, quercetin and kaempferol.

The observed antibacterial activity of our crop residue extracts could be due to the flavonoids; even though they could be found in small amounts, they exhibit membrane-disrupting activities. The mechanism of interaction involves the specific binding of flavonoids with the polar head groups of membrane lipids and non-polar compounds inside the membrane, as well as non-specific interactions of flavonoids and phospholipids that change the thickness and fluctuations of the membrane and, therefore, indirectly modulate the distribution and/or function of membrane proteins. In this way, binding to the lipid bilayer and inactivation and inhibition of intracellular and extracellular enzymes synthesis results in bacterial cell membrane damage and increased permeability (Górniak et al., 2019). In addition, tannins found in crop extracts can exhibit an antibacterial effect due to interactions with proteins in the bacterial cell wall, formation of stable water-insoluble protein components, and interfering with protein synthesis (Si et al., 2012). Si et al. (2012) found that tannins obtained by ethanolic extraction from agricultural by-products inhibited the growth of several pathogenic bacteria, including *L. monocytogenes*, *E. coli*, and Methicillin- and Vancomycin Resistant *S. aureus*.

In accordance with the present results, Alexandrino et al. (2021) did not report the bactericidal effect of the sunflower seed flour ethanolic extract against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* in the maximum concentration used (39.8 mg eq CGA/mL). On the other hand, Martin et al. (2012) showed that out of seven ethanolic extracts of different agro-industrial wastes, six had a bactericidal effect against *E. coli* and five against *L. monocytogenes*, with the observation that the bactericidal potential was lower than the inhibitory one so that, e.g., the MBC value of the most effective extract (guava bagasse) against *L. monocytogenes* was eight times higher than the MIC value.

## CONCLUSION

The results of our study indicate that ethanolic extract of wheat, sunflower, and maize crop residues possess bacteriostatic activity against some of the most common foodborne pathogens. This preliminary investigation suggests that non-edible plant parts biomass, mainly consisting of lignocellulose, has the potential as a low-value renewable source for the extraction of bioactive compounds. However, for a better understanding of the mechanism of action of the examined crop residue extracts, further research is required to precisely determine the chemical composition and identify those phenolic compounds



to which antimicrobial activity can be attributed. For the appropriate exploitation of such residues in the production of added value compounds, optimizing the extraction process and applying methods that would allow the subjection of a large amount of agricultural residues to treatment with minimal environmental impact and therefore to obtain more extract in a cost-effective way is necessary. In the context of crop residue extracts as potential natural antioxidants and antimicrobial agents that could be alternatives to synthetic additives in foods, especially in meat products, research should be directed towards consumer protection in terms of determining whether such extracts are safe for consumption, and later to develop technological processes for meat product reformulation by using such new additives with a deviation in sensory characteristics of the products that would be acceptable to consumers.

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## Author's Contribution:

MG and MBC - made equal contributions to conceptualization, data curation, formal analysis, investigation and methodology regarding MIC and MBC analysis and wrote an original draft. NČ carried out validation, visualization, and revised the manuscript. MŽB was involved in supervision, funding acquisition and revised the manuscript critically. JV performed microbiology analysis, revised and edited the draft. SS carried out plant material collection, the extracts preparation, reviewed and edited the manuscript. ZM made contributions to conceptualization, project administration, funding acquisition, supervision, review and editing of the draft.

## Competing interest

The authors declare no conflicts of interest.

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## HAEMATOLOGICAL ALTERATIONS IN CALVES WITH ACUTE RESPIRATORY DISTRESS SYNDROME DUE TO ASPIRATION PNEUMONIA: A PROSPECTIVE STUDY

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### Abstract

The aim of this prospective study was to investigate the arterial blood gas (ABG) analysis, which is considered the gold standard, and complete blood count (CBC) as a complementary test in neonatal calves with acute respiratory distress syndrome (ARDS) due to aspiration pneumonia. Ten healthy and 20 calves with ARDS due to aspiration of milk/colostrum were enrolled in the study. Clinical examinations were performed at admission. ABG analyses were performed to determine the presence of ARDS and investigate the extent of lung ventilation/damage. CBC analysis was performed from venous blood samples. Heart and respiratory rates and body temperature values were higher in diseased calves than healthy ones ( $p < 0.000$ ). Arterial pH, partial pressure of oxygen in arterial blood ( $\text{PaO}_2$ ) and saturation of oxygen in arterial blood ( $\text{SaO}_2$ ) were lower ( $p < 0.001$ ) in the diseased calves, while partial pressure of carbon dioxide in arterial blood ( $\text{PaCO}_2$ ) and lactate levels were higher ( $p < 0.000$ ) than those in healthy ones. Also, leukocyte (WBC), lymphocyte, monocyte, granulocyte, mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH) levels were higher in the diseased calves than the healthy calves ( $p < 0.032$ ). As a result of the correlation analysis, the only correlation was determined to be between  $\text{PaO}_2$  and WBC, which was moderately negative. As a result, it was observed that leukocytosis developing in respiratory diseases that cause severe inflammatory processes such as aspiration pneumonia was negatively correlated with  $\text{PaO}_2$  and could potentially exacerbate hypoxia,

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and ABG evaluation with CBC could provide useful clinical data in calves with ARDS due to aspiration pneumonia.

**Key words:** arterial blood gas, complete blood count, aspiration pneumonia, diagnosis, calf

## HEMATOLOŠKE PROMENE KOD TELADA SA AKUTNIM RESPIRATORNIM DISTERS SINDROMOM ZBOG ASPIRACIONE PNEUMONIJE: PROSPEKTIVNA ISTRAŽIVANJA

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### Kratak sadržaj

Cilj ove prospektivne studije bio je istraživanje analize arterijskih gasova krvi (AGK) koja se smatra zlatnim standardom, kao i kompletne krvne slike (CBC) kao dopunskog testa kod teladi u neonatalnom periodu sa akutnim respiratornim distresnim sindromom (ARDS) usled aspiracione pneumonije. Ispitivanje je obuhvatilo 10 zdrave i 20 teladi sa ARDS izazvane aspiracijom mleka/kolostruma. Klinički pregledi su izvršeni prilikom prijema životinja. AGK analize su izvršene da bi se odredilo prisustvo ARDS i ispitao kapacitet plućne ventilacije odnosno stepen njenog oštećenja. Analiza CBC vršena je iz uzoraka venske krvi. Vrednosti srčane i respiratorne frekvencija bile su više kod obolele teladi nego kod zdrave ( $p < 0.000$ ). Arterijski pH, parcijalni pritisak kiseonika u arterijskoj krvi ( $\text{PaO}_2$ ) i saturacija kiseonika u arterijskoj krvi ( $\text{SaO}_2$ ) bile su niže ( $p < 0.001$ ) kod obolele teladi, dok su vrednosti parcijalnog pritiska ugljen dioksida u arterijskoj krvi ( $\text{PaCO}_2$ ) kao i nivoi laktata bile više ( $p < 0.000$ ) kod obolelih jedinki u odnosu na zdrave. Pored toga, nivoi leukocita (WBC), limfocita, monocita, granulocita, srednjeg korpuskularnog volumena (MCV) i srednjeg korpuskularnog hemoglobina (MCH) bili su viši kod obolele nego kod zdrave teladi ( $p < 0.032$ ). Na osnovu rezultata korelacione analize jedina korelacija je utvrđena između  $\text{PaO}_2$  i WBC, koja je procenjena kao umereno negativna. Shodno tome, ustanovljeno je da je leukocitoza, koja se razvija u okviru respiratornog oboljenja i izaziva teške inflamatorne procese kao što



je aspiraciona pneumonija, u negativnoj korelaciji sa  $\text{PaO}_2$  i potencijalno može da pogorša hipoksiju. Analiza arterijskih gasova krvi zajedno sa CBC mogu pružiti korisne kliničke podatke za telad sa ARDS izazvane aspiracionom pneumonijom.

**Ključne reči:** arterijski gasovi krvi, kompletna krvna slika, aspiraciona pneumonija, dijagnoza, tele

## INTRODUCTION

One of the most frequent causes of economic loss in livestock is respiratory disease, which has a multifactorial origin and leads to clinical and pathological abnormalities in the respiratory system. In addition to infectious causes, aspiration pneumonia (AP) is caused by the intake of foreign substances such as milk, colostrum, liquid supplements, or medications that damage the lung tissue as a result of inappropriate drenching methods/bottles or forced feeding (Hattab et al., 2022). Due to the irritating properties of the aspirated material, gangrenous bronchopneumonia may develop in cases where substantial amounts of fluid have been aspirated, albeit death is nearly always immediate (Akyüz et al., 2022). The initiation of clinical symptoms is frequently subtle since clinical and pathological characteristics of aspiration pneumonia are highly variable (Lopez and Martinson, 2017; Hattab et al., 2022).

Lung endothelial and epithelial damage develops as a result of aspiration/inhalation of foreign matter. After aspiration of liquid substances, acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) may develop in cases where gas exchange is impaired (Ider et al., 2022). The pulmonary alveolar and bronchial epithelium are also disrupted in cases of ARDS (Osaka et al., 2011) which is characterised by hypoxia ( $\text{PaO}_2 < 60$  mmHg), respiratory acidosis, hypercapnia ( $\text{PaCO}_2 > 45$  mmHg), tachypnea (respiratory rate  $> 45/\text{min}$ ), and abdominal respiration with wheezing (Bleul, 2009; Yıldız and Ok, 2017).

Complete blood count (CBC) and arterial and/or venous blood gas measurements are primary clinical diagnostic tools. Although the findings have been reported to be non-specific, CBC analysis is recommended in cases of aspiration pneumonia (Kogan et al., 2008). The use of arterial blood samples has been suggested in the evaluation of lung ventilation (Bleul et al., 2007). However, the technical difficulty of obtaining arterial blood samples under farm conditions in veterinary medicine causes the preference for venous blood samples (Nagy et al., 2002). Therefore, the aim of this prospective study is to investigate arterial blood gas (ABG) analysis, which is considered the gold

standard in assessing lung oxygenation and ventilation-perfusion, and venous CBC findings as a complementary test and their correlation with each other in calves with ARDS due to aspiration pneumonia, and to acquire clinical information about the extent of the damage from venous blood samples in cases where arterial blood samples cannot be obtained.

## **MATERIAL AND METHODS**

This study was approved by the decision of the Ethics Committee of the Faculty of Veterinary Medicine of Harran University, with session number 2021/004, dated 07.05.2021 and numbered 01-08. All institutional and national guidelines for the care and use of study animals were followed. All calf breeders gave their consent before the commencement of the study.

### ***Animal Selection***

Twenty neonatal calves of Holstein breed, whose developed non-specific respiratory disease symptoms such as cough, nasal and/or ocular discharge, tachypnea, and respiratory distress after being fed on milk/colostrum with inappropriate methods/bottles constituted the Diseased Group. Ten healthy neonatal calves of Holstein breed which were determined to be healthy in clinical and laboratory examinations (Dillane et al., 2018), constituted the Healthy Group. Calves from dystocia, prematurity, congenital abnormalities, and those with diarrhoea and infection suspicion were excluded from the study. In addition, the formation of aspiration pneumonia due to inhalation of different liquid materials such as digestive stimulants or vitamin-mineral complex solutions was not included in the study as the alteration and severity of the investigated parameters may be affected by the character and acidity of the aspirated material. All calves included in the study were treated with appropriate treatment protocols (intravenous fluid therapy, antimicrobial, anti-inflammatory, mucolytic and intranasal oxygen administrations) following clinical examination and sampling. However, the follow-ups of the diseased calves could not be performed due to communication and/or economic issues.

### ***Clinical examinations***

Clinical examinations were performed both to detect the presence of pneumonia and to determine the present medical condition and included evaluation of hydration status, palpable lymph nodes and mucous membranes,

measurements of heart and respiratory rate, and heart and lung auscultation. In addition, thoracic ultrasonography was performed to detect the presence of pneumonia in all the diseased calves. Using a 5 MHz micro-convex probe (Mindray Z60, China), the area of interest was wetted with 70% isopropyl alcohol without clipping the hair and scanned from the 10<sup>th</sup> intercostal space towards the cranial. Clinical and ultrasonographic examinations were performed by the same veterinary staff applying the same examination protocols.

### ***Blood Sampling***

Venous blood samples (5 mL) were taken once from vena jugularis at the time of admission from all the calves included in the study. The time of blood sampling, and thus the first admission to the hospital and the onset of the symptoms, was a median of 5 (1 – 12) hours after inappropriate colostrum/milk drenching. Arterial blood samples (1 mL) were taken by a puncture of arteria auricularis caudalis, as previously reported (Nagy et al., 2002). After the sampling, it was ensured that there were no air bubbles and inadequate or excessive anticoagulant. After the sampling, the specimen was analysed immediately. For CBC analysis, venous blood samples (using tubes with K<sub>3</sub>EDTA), and for ABG analysis, arterial blood samples were used to determine the presence of ARDS and investigate the lung ventilation/damage.

### ***Arterial Blood Gas Analysis***

Arterial blood pH, partial pressure of carbon dioxide in arterial blood (PaCO<sub>2</sub>), partial pressure of oxygen in arterial blood (PaO<sub>2</sub>), saturation of oxygen in arterial blood (SaO<sub>2</sub>), and lactate measurements were performed using an automatic blood gas analyzer (epoc® Blood Analysis System, Siemens, Germany).

### ***Complete Blood Count Analysis***

Total leukocyte (WBC), lymphocyte (Lym), monocytes (Mon), granulocyte (Gra), erythrocyte (RBC), mean corpuscular volume (MCV), hematocrit (Hct), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and haemoglobin (Hb) measurements were performed using an automatic cell counter (pocH-100i, Sysmex®, Japan).

### ***Criteria for definition of Acute Respiratory Distress Syndrome***

The criteria for the presence of ARDS were hypoxia ( $\text{PaO}_2 < 60$  mmHg), respiratory acidosis, hypercapnia ( $\text{PaCO}_2 > 45$  mmHg), tachypnea (respiratory rate  $> 45/\text{min}$ ), and abdominal respiration with wheezing (Bleul, 2009). Among these parameters, the presence of at least two criteria along with  $\text{PaO}_2 < 60$  mmHg was taken into consideration.

### ***Statistical Analysis***

Statistical software (SPSS 25.00, IBM®, USA) was used to evaluate the data. We used a one-sample Kolmogorov-Smirnov test to determine whether the variables had normal distributions. Non-parametric data were evaluated by Mann-Whitney U test, and presented as median (min, max). A Spearman's correlation was performed to determine the relationship between ABG and venous CBC results. Sizes of correlation were considered 0.5 – 0.7 (-0.5 - -0.7) moderately positive (negative), 0.7 – 0.9 (-0.7 - -0.9) highly positive (negative), and 0.9 – 1 (-0.9 - -1) very highly positive (negative). Statistical significance was considered as  $p < 0.05$  and  $p < 0.01$ .

## **RESULTS**

### ***Anamnestic Data***

Anamnestic data revealed that all the calves included in the Diseased Group developed respiratory disease-related symptoms after forced feeding on milk/colostrum with inappropriate methods/bottles. The samples were taken during the calving season, between June and August. Also, all the calves of the present study, which were housed in individual pens, were naturally born ( $> 280$  days gestation) on the farm and were able to stand within 1 hour of life. The body weights of the calves in the groups were similar (Table 1). All the calves were given 2 litres of milk/colostrum within the first 6 hours of their life. The time of admission for all calves to the hospital was the day they were born. Considering the first 6 hours of colostrum administration, the mean time to hospital admission was 11 (7 - 20) hours after delivery. The complete history of each diseased calf is visualized (Figure 1.) as the symptom onset is sudden in cases of aspiration pneumonia.

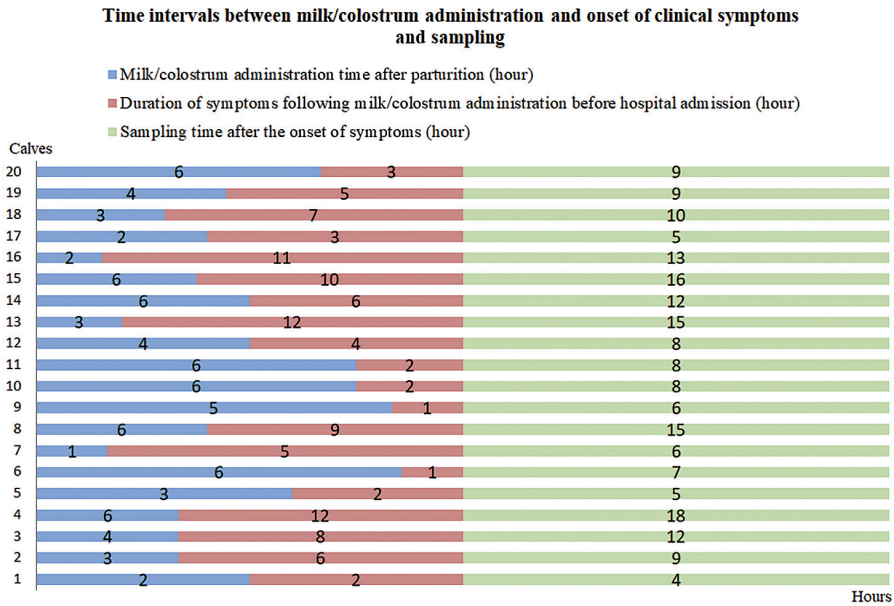


Figure 1. The complete history of time intervals of each diseased calf

### ***Clinical Examination Findings***

Clinical examinations revealed findings to suspect aspiration pneumonia, such as extended neck (14 out of 20), bilateral mucoid/purulent nasal discharge (8 out of 20), a painful expression (13 out of 20), and widespread crackles over the affected lung during auscultation (20 out of 20). In addition, as a result of the thoracic ultrasonographic examination, poorly defined lung surface dorsally (12 out of 20), B-lines origination from pleura > 3 lines (20 out of 20), and loss of A-lines (14 out of 20) were detected (Figure 2). The results of clinical examination findings of the healthy animals and the calves with aspiration pneumonia are presented in Table 1. The heart and respiratory rates and body temperature of the calves with aspiration pneumonia were significantly higher than the healthy calves ( $p < 0.000$ ).

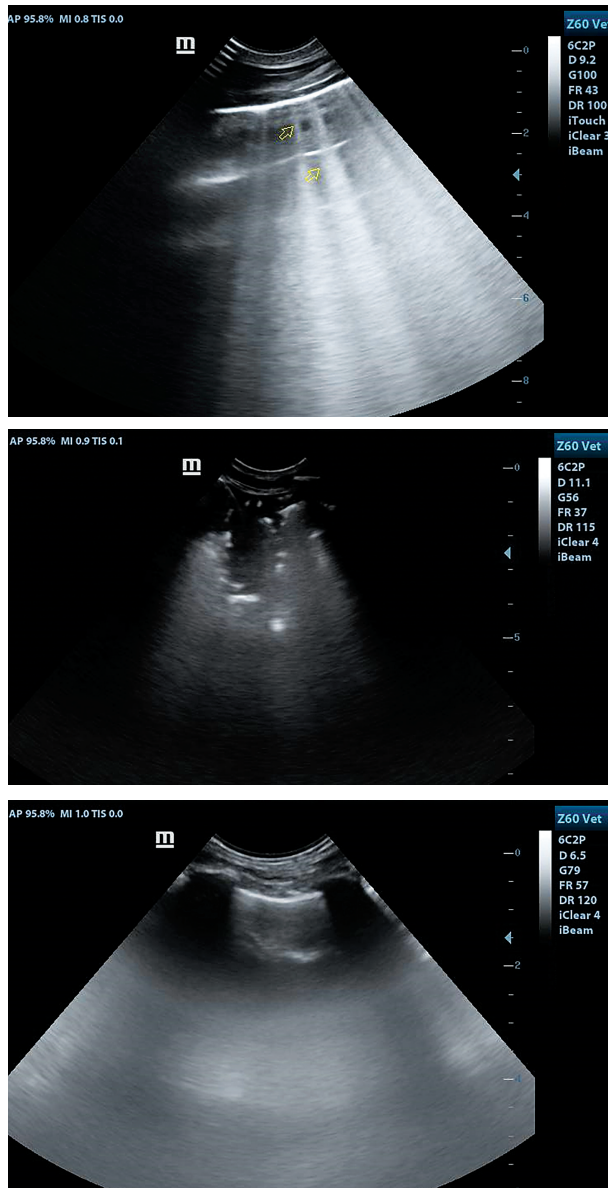


Figure 2. Thoracic ultrasonography examination findings (A. More than 3 B-lines in number, in confluent view, indicating loss of peripheral lung aeration due to interstitial disease involvement.; B. The ill-defined lung surface and anechoic area showing possibly aspirated fluid material.; C. The loss of the A-lines between the acoustic shadows formed by the ribs)

Table 1. Clinical examination findings

Parameters	Healthy Group (n=10)					Diseased Group (n=20)					P value
	min	Q1	me- dian	Q3	max	min	Q1	me- dian	Q3	max	
Body weight (kg)	42	43.75	48	51.75	55	34	39.75	47	50.25	54	0.154
Heart rate (beats/min)	86	88	99	110	120	78	140	148	160	178	0.000
Body tem- perature (°C)	37.6	38.07	38.5	39.02	39.4	38.8	39.8	40.05	40.6	41.2	0.000
Respiratory rate (breaths/ min)	36	38	42	50.5	55	52	64	74.5	77	90	0.000

### ***Arterial Blood Gas Analysis***

The results of ABG analysis findings of the healthy calves and the calves with aspiration pneumonia are presented in Table 2. Statistically significant differences were observed in pH, PaCO<sub>2</sub>, PaO<sub>2</sub>, SaO<sub>2</sub>, and lactate levels. While the pH, PaO<sub>2</sub>, and SaO<sub>2</sub> levels of the calves with aspiration pneumonia were lower than those in the healthy calves ( $p < 0.001$ ), PaCO<sub>2</sub> and lactate levels were significantly higher ( $p < 0.000$ ).

Table 2. Arterial blood gas analysis findings of the healthy and the diseased calves

Parameters	Healthy Group (n=10)					Diseased Group (n=20)					<i>p</i> value
	min	Q1	me- dian	Q3	max	min	Q1	me- dian	Q3	max	
pH	7.35	7.36	7.38	7.41	7.45	6.81	7.12	7.26	7.37	7.44	0.001
PaCO <sub>2</sub> (mmHg)	20.6	20.95	26.8	32.65	35.8	37.4	44.07	54.1	60.97	87.9	0.000
PaO <sub>2</sub> (mmHg)	79.7	82.8	85.25	89.6	93.8	8.6	22.4	25.65	31.15	41.6	0.000
SaO <sub>2</sub> (%)	86.5	90.92	95.5	90.55	99.6	5.8	26.75	39.2	43.67	53.9	0.000
Lactate (mmol/L)	0.2	0.3	0.62	0.9	0.92	1.13	2.65	5.01	7.49	13.33	0.000

pH: Power of hydrogen, PaCO<sub>2</sub>: Partial pressure of carbon dioxide in arterial blood, PaO<sub>2</sub>: Partial pressure of oxygen in arterial blood, SaO<sub>2</sub>: Saturation of oxygen in arterial blood. Min: Minimum, Q1: First quartile, Q3: Third quartile, Max: Maximum.

### Complete Blood Count Analysis

The results of the CBC analysis of the healthy calves and the calves with aspiration pneumonia are presented in Table 3. As a result of CBC analysis, it was determined that WBC, lymphocyte, monocyte, granulocyte, MCV, and MCH levels of the calves with aspiration pneumonia were higher than that of the healthy ones ( $p < 0.000$ ,  $p < 0.012$ ,  $p < 0.028$ ,  $p < 0.000$ ,  $p < 0.032$  and  $p < 0.025$ , respectively).

Table 3. CBC findings of the healthy and the diseased calves

Parameters	Healthy Group (n=10)					Diseased Group (n=20)					P value
	min	Q1	me- dian	Q3	max	min	Q1	me- dian	Q3	max	
WBC (x10 <sup>9</sup> /L)	5.4	7.43	9.4	9.4	12.5	6.38	14.57	19.59	25.45	47.67	0.000
Lym- phocyte (x10 <sup>9</sup> /L)	1.6	2.05	3.0	4.02	4.59	0.89	2.20	4.97	11.34	31.62	0.012
Monocyte (x10 <sup>9</sup> /L)	0.33	0.37	0.49	0.55	1.02	0.08	0.42	0.65	1.15	3.34	0.028



Parameters	Healthy Group (n=10)					Diseased Group (n=20)					P value
	min	Q1	me- dian	Q3	max	min	Q1	me- dian	Q3	max	
Granu- locyte (x10 <sup>9</sup> /L)	2.9	4.17	5.26	7.08	9.37	2.14	8.98	12.86	15.81	17.83	0.000
RBC (m/ mm <sup>3</sup> )	5.62	6.41	7.96	10.12	10.62	4.05	6.19	8.19	10.54	12.83	0.782
MCV (fl)	30.5	34.52	44.85	49.62	52.5	27.8	42.7	48.65	70.77	76.7	0.032
Haemato- crit (%)	23.2	25.02	37.55	45.92	52.1	21.6	33.92	40.85	48.28	64.2	0.242
MCH (pg)	7.1	9.8	11.6	12.6	15.3	4.6	11.12	13.5	20.75	28.2	0.025
MCHC (g/dL)	16.7	19.15	26	31.97	34	14.8	24.65	27.9	30.30	58.2	0.351
Hb (g/dL)	5.9	7.6	8.4	11.97	15.4	5	8.82	11.3	13.62	17.4	0.198

WBC: Total leukocyte, RBC: Erythrocyte, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, Hb: Haemoglobin. Min: Minimum, Q1: First quartile, Q3: Third quartile, Max: Maximum.

### ***Correlation Between ABG and CBC Findings***

The results of Spearman's correlation test are presented in Table 4. The only correlation detected between ABG and CBC variables was between WBC and PaO<sub>2</sub>, which was moderately negative ( $r = -0.567$ ).

Table 4. Spearman's correlation analysis results

Param- eters	pH	PaCO <sub>2</sub>	PaO <sub>2</sub>	SaO <sub>2</sub>	Lactate	WBC	Lym	Mon	Gran	RBC	MCV	HCT	MCH	MCHC	Hb
pH	1.000	-.568**	.319	.604**	-.639**	-.091	-.072	-.062	.024	-.319	.214	-.079	.077	-.060	-.101
PaCO <sub>2</sub>		1.000	-.700**	-.784**	.723**	.427*	.240	.114	.283	.301	.025	.185	.115	.006	.247
PaO <sub>2</sub>			1.000	.852**	-.677**	-.567**	-.498**	-.250	-.438*	-.026	-.225	-.128	-.260	.127	-.060
SaO <sub>2</sub>				1.000	-.778**	-.463**	-.287	-.158	-.368*	-.063	-.086	-.008	-.161	-.018	-.046
Lactate					1.000	.399*	.177	.170	.366*	.062	.367*	.409*	.247	-.198	.134
WBC						1.000	.563**	.632**	.834**	-.146	.257	.097	.409*	.317	.268
Lym							1.000	.401*	.103	.038	-.107	-.138	-.132	-.008	-.171

Parameters	pH	PaCO <sub>2</sub>	PaO <sub>2</sub>	SaO <sub>2</sub>	Lactate	WBC	Lym	Mon	Gran	RBC	MCV	HCT	MCH	MCHC	Hb
Mon								1.000	.479**	-.095	.213	.176	.384*	.310	.322
Gran									1.000	-.330	.514**	.199	.650**	.352	.360
RBC										1.000	-.547**	.315	-.451*	-.092	.290
MCV											1.000	.500**	.723**	-.163	.197
HCT												1.000	.406*	-.210	.630**
MCH													1.000	.399*	.556**
MCHC														1.000	.487**
Hb															1.000

\*\* . Correlation is significant at the 0.01 level (2-tailed).

\* . Correlation is significant at the 0.05 level (2-tailed).

pH: Power of hydrogen, PaCO<sub>2</sub>: Partial pressure of carbon dioxide in arterial blood, PaO<sub>2</sub>: Partial pressure of oxygen in arterial blood, SaO<sub>2</sub>: Saturation of oxygen in arterial blood, WBC: Total leukocyte, Lym: Lymphocyte, Mon: Monocyte, Gran: Granulocyte, RBC: Erythrocyte, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, Hb: Haemoglobin.

## DISCUSSION

Respiratory diseases, which are considered as one of the most critical health problems of the livestock industry, are very significant due to their economic impact, and the foremost cause is pneumonia. Cattle can acquire haematogenous pneumonias; however, most cases are bronchogenic or of inhalation/aspiration origin (Gülersoy and Şen, 2017). The most common respiratory diseases in neonatal calves are reported as postnatal hypoxia-hypercapnia, pulmonary hypertension, aspiration pneumonia, and bacterial and viral pneumonias (Vallés, 2013).

It has been reported that ARDS and ALI may develop due to impaired alveolar gas exchange and hypoxia that develops following incidental aspiration in cases of aspiration pneumonia (Son et al., 2017). Clinically, calves with ARDS due to aspiration pneumonia are characterized by cough, fever, tachypnea, cyanosis, and abnormal lung sounds (Ider et al., 2022). In the present study, the respiratory rate, body temperature, and the heart rate of the calves with ARDS due to aspiration pneumonia were significantly higher than the healthy calves ( $p < 0.000$ ) (Table 1). In addition, tachypnea, weakness, lethargy, mild to moderate cyanosis, tachycardia, and fever were present in all the calves with ARDS due to aspiration pneumonia. The clinical findings of the diseased calves in this study were due to the combination of factors and septic nature

of aspiration pneumonia (McGuirk and Simon, 2008), and the development of respiratory distress (Ider et al., 2022).

Abnormal blood gas alterations such as hypoxia, hypercapnia, and respiratory acidosis are common findings in calves with respiratory distress (Bleul, 2009). In the present study, pH, PaO<sub>2</sub>, and SaO<sub>2</sub> levels of the calves with ARDS due to aspiration pneumonia were significantly lower ( $p < 0.001$ ), while PaCO<sub>2</sub> and lactate levels were higher ( $p < 0.000$ ) than that of the healthy calves (Table 2). However, PaCO<sub>2</sub> levels of some of the diseased calves (6 out of 20) were determined to be less than 45 mmHg, and this finding was thought to be related to the volume of aspirated milk/colostrum (Marik, 2001). These findings indicate that significant changes in ABG balance and elevated lactate levels in calves with ARDS were due to aspiration pneumonia associated with hypoxia, lung endothelial and epithelial damage, and impaired gas exchange (Yıldız and Ok, 2017; Ider et al., 2022).

The primary clinical diagnostic tools are CBC and arterial and/or venous blood gas measurements (Kogan et al., 2008). It was reported that respiratory diseases lead to direct lung function disturbances with alterations in gas exchange and changes in the haematological profile (Šoltésová et al., 2015). Due to the septic and acute characteristics of aspiration pneumonia, it has been reported that severe infection may develop (Dhillon et al., 2020). In the present study, WBC, lymphocyte, monocyte, granulocyte, MCV, and MCH levels of the calves with ARDS due to aspiration pneumonia were significantly higher than that of the healthy calves ( $p < 0.032$ ) (Table 3). Elevated WBC, lymphocyte, monocyte, and granulocyte levels are indicators of an inflammatory process (Šoltésová et al., 2015). In the previous reports, differences in RBC count, haemoglobin and haematocrit levels were reported. These findings could be a consequence of the adaptation and compensatory processes reflecting an existing pulmonary disease, hypoxaemia, and the stimulation of erythropoiesis (Hanzlicek et al., 2010; Fraser et al., 2014). In a study in rats, hypoxia was determined by five indicators: WBC, granulocyte and RBC counts, reticulocyte count/percent, and MCH levels (Kondashevskaya et al., 2021). However, in the present study, RBC, haematocrit, MCHC and Hb levels of the diseased calves were not statistically different from the healthy ones. These findings may be related to the fact that these compensation mechanisms have not been activated yet. An elevation in MCV level may result from reticulocytosis, which is quite uncommon in chronic respiratory diseases, but in acute ones (Tsantes et al., 2004). Therefore, higher MCV and MCH levels of the diseased calves in this study may be associated with hypoxia tolerance and acuteness of the cases (Dzhalilova and Makarova, 2020).

Since blood serves as the primary channel for transferring CO<sub>2</sub> in the opposite direction and O<sub>2</sub> from the lungs to tissues, studies aimed at identifying the effects and/or reflections of hypoxia mostly on blood (Kondashevskaya et al., 2021). That is why the use of arterial blood samples has been suggested in evaluating lung ventilation/damage (Bleul et al., 2007). During infectious or non-infectious conditions causing hypoxia, erythroid cells continue performing their specific functions, changing their numbers, size, or O<sub>2</sub> content. Thus, under hypoxic conditions, WBC counts in small laboratory rodents, mainly neutrophils, are reported to be elevated (Kondashevskaya et al., 2021). As a result of the correlation analysis, which is one of the main topics of the present study, a moderate negative correlation was determined between PaO<sub>2</sub> and WBC count (Table 4). This finding may demonstrate that leukocytosis developing due to aspiration pneumonia may have a possible negative contribution to the exacerbation of hypoxia and could worsen the clinical manifestation as a consequence of rapid oxygen consumption by the leukocytes (Chillar et al., 1980). Therefore, since aspiration pneumonia in calves is an often-fatal emergency (Hattab et al., 2022), it may be considered to evaluate CBC as a complementary test along with ABG, at least for the initial assessment, and the prediction of the clinical outcome (McKeever et al., 2016).

The major limitation of the present study is the limited number of animals that may influence the significance and correlations of some investigated blood parameters. The authors recommend evaluating the present results with a larger number of animals. In addition, the lack of histopathological examination indicating lung damage and loss to follow-ups due to social or structural reasons can be considered limitations. Although the results of the present study are promising, evaluation of ABG and CBC findings along with serum biochemistry parameters may allow more accurate and faster assessment of emergency aspiration pneumonia cases based on a routine analysis.

## CONCLUSION

Aspiration pneumonia, which is often caused by inappropriate drenching and/or forced feeding, is a frequent condition that causes hypoxia, lung endothelial and epithelial damage, ARDS, and even sudden death depending on the amount of aspirated liquid. It emerged that significant changes occur in ABG and venous CBC findings of neonatal calves with ARDS due to aspiration pneumonia. It was observed that leukocytosis that develops in respiratory diseases such as aspiration pneumonia which causes a severe inflammatory process, could potentially exacerbate hypoxia. As a result, it was concluded

that CBC analysis with ABG measurement findings could provide valuable clinical data in calves with ARDS due to aspiration pneumonia.

### **Author's Contribution**

EG and CB made contributions to conception, methodology, involved in data collection, formal analysis and drafting the manuscript. İG and AŞ carried out the data collection and drafting of the manuscript. All authors revised the manuscript critically and EG together with CB prepared the final draft of the manuscript etc. All authors read and approved the final manuscript.

### **Competing interest**

The authors declare that they have no competing interest.

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## ANTIMICROBIAL RESISTANCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE* FIELD ISOLATES IN AUTONOMOUS PROVINCE OF VOJVODINA, REPUBLIC OF SERBIA

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### Abstract

*Actinobacillus pleuropneumoniae* (APP) is one of the most important bacterial respiratory pathogens in swine. It is the only etiological agent of porcine pleuropneumoniae (PPP) or appears as a secondary bacterial infection of the swine's respiratory disease complex (PRDC). Antibiotics are still the most effective measure for reducing mortality and severity of clinical symptoms in most parts of the world. Nevertheless, in recent years, resistance to certain antibiotics such as tetracycline, penicillin, aminoglycosides and others has been increasing. The aim of this study was to examine the resistance of *A. pleuropneumoniae* isolates in the area of AP Vojvodina. The samples were collected from dead pigs' lung tissue from 14 farms. Bacterial strains were isolated on agar with 5% sheep blood and *Staphylococcus aureus* culture as a source of factor V and for subculture chocolate agar enriched with 10 mg/L NAD was included as well. After the examination of morphological and cultural characteristics, biochemical identification was performed using standard bacteriological tests. APP was confirmed by PCR. A total of 35 bacterial isolates were tested on antimicrobial resistance using the disc diffusion method with 11 antibiotics. Antibiotics from the group: aminoglycosides, sulfonamides, fluoroquinolones, phenicols, tetracyclines and beta-lactam antibiotics were used. The isolates collected in the area of AP Vojvodina, from 2015 to 2022, retained high sensitivity to florfenicol, cephalosporins and fluoroquinolones. However, increasing

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antibiotic resistance was observed for penicillin, tetracycline and all tested aminoglycosides.

**Key words:** *A. pleuropneumoniae*, antibiotics, resistance, swine

## ANTIMIKROBNA REZISTENCIJA IZOLATA *ACTINOBACILLUS PLEUROPNEUMONIAE* NA TERITORIJI AUTONOMNE POKRAJINE VOJVODINE, REPUBLIKA SRBIJA

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### Kratak sadržaj

*Actinobacillus pleuropneumoniae* (APP) se ubraja u najznačajnije bakterijske respiratorne patogene svinja. Jedini je etiološki agens *porcine pleuropneumoniae* (PPP) ili se pojavljuje kao sekundarna bakterijska infekcija u kompleksu respiratorne bolesti svinja (PRDC). Antibiotici su i dalje najefikasnija mera za smanjenje mortaliteta i težine kliničke slike u većini dijelova svijeta, međutim tokom prethodnih godina javlja sve češća pojava rezistencije na pojedine antibiotske preparate poput tetraciklina, penicilina, aminoglikozida i dr. Cilj ove studije jeste ispitati rezistenciju izolata *A. pleuropneumoniae* na prostoru AP Vojvodine. Uzorci su prikupljeni iz plućnog tkiva uginulih svinja sa 14 farmi. Za izolaciju *A. pleuropneumoniae* korišten je agar sa 5% ovčije krvi i kultura soja *Staphylococcus aureus* kao izvor V faktora, a za presejavanje čokoladni agar obogaćen sa 10 mg/L NAD. Nakon ispitivanja morfoloških i kulturelnih karakteristika izvršena je biohemijska identifikacija primenom standardnih bakterioloških testova. APP je potvrđen pomoću PCR metode. U ispitivanju antibiotske osetljivosti 35 bakterijskih izolata korištena je disk difuzionna metoda sa ukupno 11 antibiotika. Korišteni su antibiotici iz grupe; aminoglikozida, sulfonamida, fluorohinolona, fenikola, tetraciklina i beta-laktamskih antibiotika AB preparata. Izolati prikupljeni na teritoriji AP Vojvodine od 2015 do 2022 godine zadržali su visoku osetljivost na florfenikol, cefalosporine i fluorohinolone. Kod drugih antimikrobnih sredstava kao što su penicilin,

tetraciklin i svi testirani aminoglikozidi, uočena je povećana rezistencija na antibiotike.

**Ključne reči:** *A. Pleuropneumoniae*, antibiotici, rezistencija, svinje

## INTRODUCTION

Swine pleuropneumonia is one of the most important swine bacterial respiratory pathogens occurring worldwide. The occurrence of acute epidemics characterized by high mortality results in economic damage, loss in production, reduced growth and high medical costs. Many herds get infected with several strains. High virulence strains can be present in a herd for a long period of time without clinical symptoms or lesions in the slaughterhouse.

The causative agent of the disease, *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) is a small, Gram-negative, encapsulated rod with typical coccobacillary morphology. *A. pleuropneumoniae* isolates can be classified into two biovars depending on the need for nicotinamide dinucleotide phosphate (NADP) for growth, with biovar 1 dependent on NADP and significantly less common biovar 2 being independent of NADP (Gottschalk, 2012). There are currently 19 recognized serovars of *A. pleuropneumoniae*, based on their capsule synthesis genes (*cps*), with certain differences in geographical distribution and pathogenicity (Bossé et al., 2018; Sassu et al., 2018). In most European countries, serovar 2 is a dominant strain causing most disease outbreaks (Sárközi et al., 2018). This was confirmed recently in a German study based on more than 200 APP isolates originating from the same geographical area from the years 2010–2019, where 64% were found to belong to serovar 2 as the predominant serotype (Schuwerk et al., 2021). The most important virulence factors of *A. pleuropneumoniae* are capsular polysaccharides, LPS (endotoxin), and outer membrane proteins and exotoxins (Apx - toxins). *A. pleuropneumoniae* produces protein cytotoxins ApxI, ApxII, ApxIII and ApxIV belonging to the so-called RTX toxin family, which secrete different serotypes in various combinations, through type 1 secretory mechanism (Schaller et al., 2000).

Antimicrobial resistance is becoming a major problem both in Serbia and worldwide. The aim of this study is to examine the susceptibility of pathogens to antibiotics that are most commonly used in the treatment of this disease. Infections in humans caused by antimicrobial resistance bacteria of animal's origin is becoming an urgent threat to the control of bacterial infections. Identification of antibiotic-resistant or susceptible strains is essential in the fight

against antibiotic-resistant pathogens. Determination of susceptibility to antimicrobial drugs is performed by disk diffusion method or by determining the minimum inhibitory concentration (MIC).

MATERIAL AND METHODS

The isolates used in this study were from lung tissue of pigs' carcasses from 14 different farms in the territory of AP Vojvodina (Figure 1). Lung tissue samples were collected from carcasses with typical lesions on the lungs (elevated cherry-coloured red areas/red areas of haemorrhagic-necrotic pneumonia) with pronounced interlobular edema and covered with fibrin deposits.



Figure 1. Distribution of *A. pleuropneumoniae* isolates in the territory of AP Vojvodina (The map was downloaded from <https://www.superjoden.nl/regioni-srbije-mapa.html>)

*A. pleuropneumoniae* isolates were plated on Columbia Blood Agar with 5% defibrinated sheep blood. Culture of *Staphylococcus aureus* strain was used as a source of factor V and for subculture chocolate agar with PolyVitex (BioMerieux, Marcy l'Etoile, France) enriched with 10 mg/L NAD was included as well. Incubation lasted for 24 h at 37 °C in the presence of 5% CO<sub>2</sub>. After examination of morphological and cultural characteristics, biochemical identification was performed using standard bacteriological tests (Žutić et al., 2009).

APP was also confirmed by PCR. Five pairs of oligonucleotide primers described by Rayamajhi et al., 2005, are used for the amplification of 4 "Apx" genes encoding exotoxin synthesis ("ApxI", "ApxII", "ApxIII" and "ApxIV") characteristic of all serotypes and biovars of *A. pleuropneumoniae*. Amplification of the appropriate parts of the genome are performed using the polymerase chain reaction (PCR) in multiplex format (mPCR) using the set for performing the mPCR reaction (EURx). A "primer-mix" is prepared for a total reaction volume of 50 µl, with an oligonucleotide-primer concentration of 0.5 µM and a maximum number of oligonucleotide-primer pairs of 5 per one reaction according to the following formula: 25 µl master mix + 25 µl (primer mix + sample).

Urease activity and positive Christie-Atkins-Munch-Peterson (CAMP) test further refer to *A. pleuropneumoniae*. From fresh clinical samples, 70% of the strains were 'sticky' and difficult to remove from the plate, which correlates with their ability to form a biofilm in vitro.

A total of 85 isolates were isolated from 14 farms across AP Vojvodina (Figure 1). Thirty-five isolates were tested for their susceptibility to a panel of antimicrobials by disk diffusion method on Mueller Hinton Agar Plate (MH agar) with the addition of 5% defibrinated horse blood for fastidious bacteria. The following antibiotics were used: penicillin G (6 mg), amoxicillin (25 mg), ampicillin (10 mg), ceftiofur (30 mg), trimethoprim/sulfamethoxazole (1.25 mg / 23.75 mg), enrofloxacin (5 mg), florfenicol (30 mg), gentamicin (15 mg), neomycin (30 IU), streptomycin (10 IU), tetracycline (30 IU). The isolates were classified as S-sensitive, I-intermediate, and R-resistant. In the assessment of resistance, intermediate isolates were considered resistant. Interpretation of growth inhibition followed the clinical breakpoints approved by the Clinical Laboratory Standards Institute (CLSI 2018).

## RESULTS AND DISCUSSION

Altogether, 51.4% of isolates were susceptible to tetracyclines. Less than half of the isolates, more precisely, 48.6% were sensitive to penicillin. The ef-

ficacy results within the aminoglycoside group differ markedly. Only 28.6% of isolates were sensitive to streptomycin and 34.3% to neomycin. Gentamicin, on the other hand, shows effectiveness in 65.7% of isolates (Table 1.). Our results show an identical efficiency of 74.3% and a low rate of resistance to fluoroquinolone (enrofloxacin) and sulpha drug. With 80% of sensitive isolates, 3<sup>rd</sup> generation cephalosporin was in the second place in terms of efficacy. Florfenicol shows best results and the lowest rates of antimicrobial resistance. Only 17.1% of isolates were resistant (including intermediates).

Table 1. Resistance of 35 *A. pleuropneumoniae* isolates found in the territory of AP Vojvodina

Antibiotic	S- sensitive (%)	I-intermediate (%)	R-resistant (%)
Penicillin G	48.6	0	51.4
Ampicillin	68.6	2.8	28.6
Amoxicillin	68.6	5.7	25.7
Tetracycline	51.4	8.6	40
Ceftiofur	80	8,6	11.4
Gentamicin	65.7	5.7	28.6
Neomycin	34.3	17.1	48.6
Streptomycin	28.6	17.1	54.3
Trimethoprim/sulfamethoxazole	74.3	0	25.7
Enrofloxacin	74.3	11.4	14.3
Florfenicol	82.9	5.7	11.4

Antimicrobials are intensively used in swine production for therapeutic, metaphylactic and prophylactic purposes, which has led to an increased risk of bacterial resistance over time (Jensen et al., 2006; Aarestrup et al., 2008). Moreover, the movement of pigs between herds or between countries is another key factor that can contribute to the spread of antimicrobial-resistant isolates in swine populations.

Antimicrobials that are widely used in the treatment of swine pleuropneumonia were tested. Penicillin, tetracycline, amoxicillin, cephalosporin and florfenicol are recommended by the latest guidelines for pleuropneumonia therapy.

As already reported,  $\beta$ -lactams have shown high levels in vitro activity against *A. pleuropneumoniae* (Yoshimura et al. 2002; Matter et al., 2007). However, in recent years, a relatively large number of resistant isolates have

been reported, mainly penicillin, while amoxicillin and ampicillin are slightly effective. Our results confirm this rising trend mostly for penicillin. Minor antimicrobial activity of aminoglycosides against *A. pleuropneumoniae* isolates in our study are confirmed by the results in other studies such as Gutierrez-Martin (2006) and Matter et al. (2007). Tetracyclines and sulpha drugs are still among the recommended antimicrobial agents for the treatment of swine pleuropneumonia (Burch et al., 2008). However, these antimicrobials have been used extensively to treat several swine diseases over decades, so increased resistance rates have been reported in several European countries (Gutierrez-Martin et al., 2006; Hendriksen et al., 2008). Cephalosporins (3rd generation) have shown very good results. Florfenicol provides the best results and the lowest rates of antimicrobial resistance. In vitro activity of florfenicol in clinical isolates *A. pleuropneumoniae* has been extensively studied and low rates of resistance have been found in Germany, South Korea, Spain, and Japan (Gutierrez-Martin et al. 2006, Morioka et al., 2008). The fluorinated chloramphenicol derivative is a broad-spectrum antimicrobial drug, which has been licensed in Europe since 2000 for the treatment of bacterial respiratory tract infections in pigs (Kehrenberg et al., 2004).

## CONCLUSION

Isolates collected from the territory of AP Vojvodina from 2015 to 2022 retained high sensitivity to certain antimicrobial drugs used in therapy of *A. pleuropneumoniae* such as florfenicol, cephalosporin and fluoroquinolones. With other antimicrobials such as penicillin, tetracycline and aminoglycosides, increasing antibiotic resistance is observed. This confirms the importance of continuous monitoring of clinical isolates. In order for therapy to be successful and the use of antibiotics rational, it is necessary to have reliable data on the antimicrobial resistance for any pathogenic microorganisms. All farms should perform an antibiogram test before treating pleuropneumonia. It is an additional cost, but it pays off in the eventually. The therapy will be more successful, and the occurrence of antibiotics resistance will be reduced.

For years, the cause of the disease and the disease itself have been studied both together and separately. The acquired knowledge has enabled the improvement of diagnostic tests, vaccines and relatively effective eradication strategies. However, the *A. pleuropneumoniae* remains a significant cause of large economic losses for the swine industry and there is more space for the improvement of control and eradication of this pathogen.



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KK and OS made contributions to the idea of the publication, organisation of work and writing the manuscript; OS reviewed the manuscript; BS gave the final approval of the manuscript to be published.

## Competing interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Case report

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## **ACUTE BOVINE MASTITIS CAUSED BY *KLEBSIELLA PNEUMONIAE* – CASE REPORT**

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### **Abstract**

This case report describes the acute mastitis with signs of endotoxemia in the 32-month-old Simmental cow, antimicrobial susceptibility of *Klebsiella pneumoniae* isolated from a milk sample, and demonstrates the effectiveness of the applied therapy. Case presentation included the cow-lying syndrome together with the presence of clinical signs of endotoxemia accompanied by milk discoloration and “clots” formation. Bacteriology finding of the *K. pneumoniae* and antibiogram pointed that the isolate was sensitive to ceftriaxone, and trimethoprim/ sulfamethoxazole, while resistant to amoxicillin, gentamicin, ampicillin, ceftiofur, and cephalexin. The selection of treatment options came from the availability of an adequate route of administration, urgency for treatment, and lack of time for assessment of other body functions. The therapy by ceftriaxone at an intravenous dose rate of 8mg/kg with adequate supportive therapy NSAID, vitamin C, and correction dehydration showed a successful effect in life-saving procedures. The outcome of *K. pneumoniae* mastitis with endotoxemia did not affect the outcome of pregnancy.

**Key words:** ceftriaxone, endotoxemia, *Klebsiella pneumoniae*, mastitis

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## AKUTNI GOVEĐI MASTITIS IZAZVAN BAKTERIJOM *KLEBSIELLA PNEUMONIAE* – PRIKAZ SLUČAJA

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### Kratka sadržaj

Ovaj prikaz slučaja opisuje slučaj akutnog mastitisa sa znacima endotoksemije kod krave simentalske rase, stare 32 meseca, antimikrobnu osetljivost *K. pneumoniae* izolovanu iz uzorka mleka i prikaz primenjene terapije. Prikaz slučaja je uključivao prisustvo sindroma ležeće krave sa prisustvom kliničkih znakova endotoksemije praćene promenom boje mleka i prisutnim ugrušcima mleka. Bakteriološki nalaz *K. pneumoniae* i antibiogram pokazali su da je izolat osetljiv na ceftriakson i trimetoprim/sulfametoksazol, dok je otporan na amoksicilin, gentamicin, ampicilin, ceftiofur i cefaleksin. Izbor opcije za lečenje je proizašao iz dostupnosti, hitnosti lečenja i nedostatka vremena za procenu ostalih telesnih funkcija. Primena ceftriaksona u intravenskoj dozi od 8 mg/kg uz adekvatnu suportivnu terapiju NSAIL, vitamin C i korekciju dehidracije pokazala je uspešan efekat u lečenju mastitisa. Ishod mastitisa *K. pneumoniae* nije uticao na ishod graviditeta.

**Ključne reči:** ceftriakson, endotoksemija, *Klebsiella pneumoniae*, mastitis

### CASE PRESENTATION

Forty percent of all clinical mastitis are caused by coliform bacteria *E. coli*, *Klebsiella spp.*, *Enterobacter spp.* (Schukken et al., 2012) with significant losses in milk production and a high economic cost of treatment (Munoz et al., 2007). The primary source of *K. pneumoniae* in dairy farms was faecal discharges, as

well as the presence of organic materials, such as wheat straw, barley straw, sand, and wood (Munoz et al., 2006), while the environmental factors, unhygienic conditions, and organic bedding are associated with the appearance of mastitis (Ngu Ngwa et al., 2018), where the opportunistic pathogens like *K. pneumoniae* additionally grow on organic and inorganic bedding materials (Zdanowicz et al., 2004). A 32-month-old pregnant Simmental cow with a daily milk yield of 25 kg (before the onset of mastitis) milking thrice per day, two months pregnant (pregnancy was confirmed by ultrasound a couple of days before mastitis onset) was presented with swollen, painful, and stiff udder. The California mastitis test (CMT) score was (+++), i.e., a severe reaction to the CMT. The milk was a watery grey colour with clots. The cow had a rectal temperature of 39.6°C and displayed the lying-cow syndrome, similar to milk fever with preserved ruminal contraction. The daily milk yield drop was drastic, to only 1 kg per 24 h. Diagnosis of mastitis was based on general clinical examination, physical examination of the udder, physical changes in the milk, the California mastitis test (CMT) score, and later findings of microbiological analysis of the milk. Hematological and biochemical blood analyses were also performed. Each mammary gland quarter sample was collected aseptically and transported to the laboratory under refrigeration (4–8°C).

After collection, the samples were placed in ice-cooled hand freezers and transported to the laboratory within 4 hours. The microbiology examination was conducted on routine methods (Markey et al., 2013). In brief: the milk samples were put on Columbia blood agar, MacConkey agar, and Sabouraud dextrose agar (all Torlak, Serbia) and kept at 37°C under aerobic conditions for 24 h, while Sabouraud agar was reincubated under aerobic conditions at 25°C up to 72 h. The primary isolate was stained by Gram procedure (Biomereux, France), followed by catalase and oxidase reaction (HiMedia, India). Presumptive diagnosis based on growth characteristics and said reactions were subjected to further biochemical testing using commercial kits for enteric non-fermenter bacteria (BBL Crystal, E/N, ID kit, Becton, Dickinson and company, USA). There was no growth on Sabouraud dextrose agar.

The antibiograms were done following the ISO 20776-1:2006 standard by disk diffusion method using antimicrobial test disks (BBL, USA) to determine susceptibility toward and other antibacterials and antibiotics: amoxicillin with clavulanic acid (20/10 µg), ampicillin (5 µg), cephalexin (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), trimethoprim+sulfamethoxazole (23.75/1.25 µg), enrofloxacin (5 µg). Interpretative zones were estimated following the standard in antimicrobial testing (CLSI).

## DISCUSSION

A bacteriology test with antibiogram revealed *K. pneumoniae* mastitis in one quarter with two antimicrobial options: ceftriaxone and trimethoprim/sulfamethoxazole. The treatment option was influenced by the availability of an adequate route of administration, treatment urgency requirement, confirmed pregnancy, and lack of time for assessment of other parameters (such as renal function, hypoglycaemia, or folate deficiency) on the animal with presumptive endotoxemia. The therapy by ceftriaxone at an intravenous dose rate of 8mg/kg with adequate supportive therapy NSAID, vitamin C, and body fluid status correction was started immediately. The clinical appearance of the udder with systemic health deterioration with the effects of endotoxemia indicated the appearance of coliform mastitis. Previous records showed a couple of *Escherichia coli* mastitis isolates on the farm, all susceptible to ceftriaxone. In addition to the presence of cow-lying syndrome, the biochemical analysis of blood showed that calcium and magnesium were at the limit values of 2.2 mmol/L and 0.9 mmol/L, respectively. The cow was treated with ceftriaxone daily during five days, and supportively with a large volume of Hartman's solution (100ml/kg body weight per 24h), glucose 10% (1000 ml per 24h), flunixin meglumine (2.2 mg/kg b.wt intravenously once daily during five days) and vitamin C (4 mg/kg b.wt intravenously once daily during three days). Three days after treatment, the CMT reaction showed only mild reaction to CMT. Five days after therapy, the CMT reaction was negative, and no clinical signs were present.

The previous history of mastitis in the herd was unrelated to the incidence of *K. pneumoniae*. The set of new wheat straw as bedding material a few days before the onset of mastitis was a potential way of introducing *K. pneumoniae* to the farm, adequate to previous findings that the presence of organic matter as bedding may be the cause of mastitis (Munoz et al., 2006).

The antimicrobial susceptibility of *this* bacteria varies between different studies due to a large number of strains (Osman et al., 2014). The present case showed the antibiogram to be sensitive to ceftriaxone and trimethoprim/sulfamethoxazole; however, other studies (Enferad and Mahdavi, 2021) established that most strains of *K. pneumoniae* were 75% and 55% resistant to ceftriaxone and oxytetracycline, respectively. The duration of treatment (5 days) and persistence of clinical signs in our case followed previously reported study (Hoe and Ruegg, 2005). Supportive treatment of fluids and electrolytes using NSAID (flunixin meglumine) reduced the clinical signs of endotoxemia and accelerated recovery. We consider supportive therapy with vitamin C to reduce the duration of mastitis because ascorbic acid affects the recovery after clini-

cal mastitis (Naresh et al., 2002). After 15 days from the completed treatment, daily milk production had increased to 20 L; at the same point, pregnancy control was performed and reperformed two months after treatment, confirming pregnancy while assuring the success of the applied therapy.

In the present case, ceftriaxone administration was justified because of the narrowed choice of effective antibiotics available. After 15 days, the cow regained 80% of its daily milk production. We conclude that correction of dehydration, executive elimination of *K. pneumoniae* systemic endotoxins, and ceftriaxone as the antibiotic of choice based on the antibiogram, assisted by vitamin C supportive therapy, resulted in a quick recovery. The outcome of mastitis in this cow or treatment option did not affect the pregnancy outcome. Educating the farmers about the potential source of infection is essential to prevent new episodes of mastitis.

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## **Author's contribution:**

MN and JŽ made contributions to conception, design of the study, and collected of samples, NZ and DG conducted the microbiological analysis, SA and ZJS involved in drafting the manuscript and analysed data, JB revised the manuscript critically and together with MN prepared the final draft of the manuscript.

## **Competing interest**

The authors declare that they have no conflict of interest.

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## ADVERSE EFFECTS OF WET DISTILLERS GRAINS WITH SOLUBLES (WDGS) IN THE DIET OF DAIRY COWS

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### Abstract

Corn wet distillers grains with solubles (WDGS) included up to 25% in the diet of 16 Simmental dairy cows, 27 to 72 months old, during three weeks, negatively affected rumen function monitored by non-invasive parameters, such as fecal consistency, and also influenced milk quality by reducing milk fat content. Dry distillers' grains with solubles (DDGS) are currently the most attractive by-product of the bioethanol industry due to the possibility of application in the diet of various animal species, whereas WDGS is often more locally available and financially affordable; however, there is little data in the literature indicating potential negative consequences of its use. Therefore, this report aims to point out, based on a practical example, the possible problems of its application in the diet of dairy cows as the most metabolically sensitive category, as well as to demonstrate steps to prevent and/or mitigate eventual errors.

**Key words:** milk fat, ruminal function, sodium bicarbonate, WDGS

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## NEGATIVNI EFEKTI SPOREDNOG PROIZVODA PROCESA PROIZVODNJE BIOETANOLA IZ KUKURUZA (DŽIBRE) U ISHRANI MUZNIH KRAVA

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### Kratak sadržaj

Vlažni sporedni proizvod destilacije alkohola iz žitarica (kukuruzna džibra) primenjen u količini do 25% u obroku 16 muznih krava simentalne rase, starosti od 27 do 72 meseca, tokom tri nedelje, negativno je uticao na funkciju buraga praćenu neinvazivnim parametrima, kao što je konzistencija fecesa, a takođe i na kvalitet mleka usled smanjenja sadržaja mlečne masti. S obzirom na to da je suva džibra trenutno najatraktivniji sporedni proizvod industrije bioetanola zbog mogućnosti primene u ishrani različitih životinjskih vrsta, ali da je vlažna kukuruzna džibra često lokalno dostupnija i finansijski pristupačnija, kao i da je u literaturi malo podataka koji ukazuju na potencijalne negativne posledice njene upotrebe, cilj ovog izveštaja je da na osnovu praktičnog primera ukaže na moguće probleme primene u ishrani mlečnih krava kao metabolički najosetljivije kategorije, kao i da demonstrira korake za preveniranje i/ili ublažavanje eventualnih grešaka.

**Ključne reči:** džibra, mlečna mast, natrijum bikarbonat, ruminalna funkcija

### CASE PRESENTATION

In the last few decades, the expansion of the production of alternative fuels from plant raw materials, traditionally intended for food, has led to significant changes in the field of the energy industry as well as agriculture and food and feed production. Worldwide, the most pronounced trend is the development of integrated technology in which by-products would be maximally valorized, thus achieving greater productivity with minimal environmental pollution. Dry distillers' grains with solubles (DDGS) currently is the most attractive by-product of the bioethanol industry due to the possibility of application in the

nutrition of cattle, pigs, poultry, and fish (Semenčenko et al., 2013). However, wet distillers' grains with solubles (WDGS) is also used. It is a product obtained after distilling alcohol and before drying to DDGS. It consists primarily of up to 70% moisture, fiber, protein, and fat. In addition to the unchanged starting substances from the raw material, it contains yeast cells and products of yeast metabolism from the fermentation process, such as B vitamins and some growth factors.

The aim of this report is to show a case of the negative effect of the substitution, in dairy cow rations, of traditional protein and starch sources with more sustainable "circular" feeds in order to increase the sustainability of dairy production. For this purpose, 16 mid-lactating (average daily milk production  $23.81 \pm 4.55$  L) Simmental dairy cows, 27 to 72 months old, were blocked and assigned to the 21-day long nutrition treatment based on the addition of WDGS as 25% of the daily meal (which consisted of 6 - 8 kg of 18% crude protein concentrate and 8 kg of silage, plus alfalfa hay ad libitum). The chemical composition of WDGS as a percent of dry matter (% DM) is shown in Table 1.

Table 1. Chemical composition of WDGS (% DM)

Moisture	Ash	Fat	Crude protein	Crude fiber
69.12	0.63	1.97	10.13	3.82

Cows selected for the trial were housed in a naturally ventilated tie-stall barn with individual feed managers and drinkers (water intake ad libitum). They were milked twice a day (08:00 a.m. and 07:30 p.m.), and the daily individual milk production was recorded. The health condition of the animals was monitored daily with particular attention to indicators of digestive disorders (rumen motility and feces appearance). At the beginning of the experiment (day 0), as well as at the end of the treatment (day 21), and on the 42<sup>nd</sup> day after a three-week break in the use of WGGs, the milk quality (the content of milk fat, protein, lactose, and non-fat dry matter (NFDm)) was assessed using standard chemical methods and registered individually for each cow. Based on the changes in the consistency of cows' excrement through the first weeks, subacute acidosis (SARA) was suspected and then confirmed by the results of the milk tests as a statistically very significant drop in fat content on day 21 compared to day 0 ( $< 0.01$ ). Daily milk yield (DMY) and protein, lactose, and NFDm content were not affected (Table 2).

Table 2. Daily milk yield (DMY) and milk composition (mean value  $\pm$  SD)

Sampling day	DMY [l/day]	Fat content [%]	Protein content [%]	Lactose content [%]	NFDM [%]
0	23.81 $\pm$ 4.55	4.03 $\pm$ 0.20 <sup>a</sup>	3.38 $\pm$ 0.12	4.66 $\pm$ 0.08	9.07 $\pm$ 0.16
21	23.38 $\pm$ 4.15	3.57 $\pm$ 0.41 <sup>b</sup>	3.43 $\pm$ 0.19	4.68 $\pm$ 0.11	9.05 $\pm$ 0.14
42	23.25 $\pm$ 3.57	4.02 $\pm$ 0.12 <sup>a</sup>	3.44 $\pm$ 0.12	4.68 $\pm$ 0.11	9.11 $\pm$ 0.11

<sup>ab</sup>Values in rows bearing different superscripts differ significantly ( $p < 0.01$ )

WGGS was removed from the diet at that moment and, although the motor function of the rumen was preserved during the whole trial, and the number of rumen contractions was within the physiological limits of 7 - 14 contractions lasting 5 minutes in all tested cows, each cow received 100 g of sodium bicarbonate daily (divided into morning and evening doses) up to 42<sup>nd</sup> day to treat subacute acidosis, i.e. to neutralize rumen acidity and correct pH. During the mentioned period, milk quality was restored, so milk analyses on the 42<sup>nd</sup> day indicated recovery of milk fat content (Table 2) and other SARA symptoms.

The basic statistical analysis was done in Excel, and a comparison of statistical significance was performed by t-Test (Paired Two Sample for Means).

## DISCUSSION

WDGS are sold prior to drying. Traditional wet distillers grains contain 30 to 35% DM and are similar in nutrient composition to DDGS, but they can vary considerably. WDGS are often lower in price on a dry matter basis compared to DDGS, but the producer must determine that WDGS can be successfully used in his/her operation. There are benefits of using WDGS, mainly because of their high palatability and how they condition very dry diets. Total mixed rations that contain 10 to 20% WDGS maintain greater homogeneity as dry particles stick together. From a practical standpoint, this results in less particle separation and less sorting by cattle. Methods to conserve and equipment to handle WDGS on the farm are among the challenges producers often face (Kalscheur and Garcia, 2019).

Despite being a potentially good option for economic and quality reasons, the high moisture content of WDGS imposes storage, transport, and high perishability constraints on farmers (Anderson et al., 2015; Moyo et al., 2016;

Ranathunga et al., 2018). Such consistency easily results in the formation of mold and quality decrease after four days under normal handling conditions (Souza et al., 2016; Moyo et al., 2016). Drying WDGS (40 – 70% moisture) to produce dried distillers' grains with solubles (DDGS, 10 – 13% moisture) is costly, and the heat processing during drying can make DDGS more susceptible to protein damage and lead to poor amino acid availability (Cao et al., 2009).

The nutrient content of DGS is influenced by factors such as the type and quality of the grain, milling, and fermentation processes. WDGS is low in effective fiber content (cellulose), which, together with excessive shredding and due to a sudden change in diet, as well as a disturbed ratio of the concentrated and roughages of the meal, leads to the development of subacute acidosis.

As explained in the paper of Kitkas et al. (2019), consequences of SARA in dairy cows may include a decrease in milk fat due to changes in rumen fermentation and fatty acids profile. Low ruminal pH alters rumen bacteria populations and fermentation patterns, favoring the production of specific long-chain fatty acids (LCFA) inhibit milk fat synthesis in the udder after absorption. Statistically highly significant ( $< 0.01$ ) drop in milk fat content between 0 and 21<sup>st</sup> day of our treatment with 25% of WDGS is not in compliance with the findings of Kavitha et al. (2021), who concluded that WDGS could be included up to 35% of DM requirement with no adverse effect on milk yield and composition of the crossbred cows for economical production. A similar positive statement was reported by Mammi et al. (2022), who found out that the rational inclusion of wheat distillers in properly formulated rations represented a safe opportunity to reduce the environmental impact of dairy farming while maintaining high production levels.

In our case, the negative impact on milk composition appeared as a consequence of the development of acidosis, which most likely occurred due to the inadequate quality of the feedstuff itself, which was used without prior analysis. After tests were carried out in order to determine the cause of the rumen disorder, it was determined that the used WDGS had an unexpectedly poor composition (Table 1) and that the appearance of acidosis most likely occurred due to the lack of effective fibers in the cow's ration (Arias et al., 2012).

This situation was overcome by the removal of WDGS from the diet of dairy cows and by simultaneous intervention with sodium bicarbonate. Rumen buffering is a common practice on dairy farms, although various products are designed for this purpose. Cruywagen et al. (2015) observed in their experiment that the inclusion of buffer in the diet of lactating cows has a positive effect on milk production and milk composition, which is in agreement with the case experience presented here.

As stated by Kalscheur and Garcia (2019) and confirmed within our farm case, when formulating animal diets, knowing the accurate nutrient composition of DGS is critical. Laboratory testing of purchased DGS is highly recommended, although only sometimes practical for some shipments. One of the challenges is finding complementary feeds that will compose an appropriate meal. Fibrous residues such as corn stover, straw, and roughages with low concentrations of total digestible nutrients are ideal feedstuffs to complement a diet that includes ethanol by-products. Blending WDGS with forages creates a complementary nutrient profile by enhancing higher physically effective fiber (Arias et al., 2012), while co-ensiling WDGS with whole-plant maize provides the opportunity for the long-term storage and utilization of WDGS (Moyo et al., 2022).

Finally, it is necessary to emphasize once again and conclude that any sudden change in the diet, especially of dairy cows, as a category with the most sophisticated metabolic requirements, implies a preliminary analysis of the ingredients and the ratio as a whole. Ad hoc reaching for more available and/or affordable solutions often costs much more.

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## **Author's Contribution:**

KN and MN made contributions to conception and design of the study, involved in data collection, and drafting the manuscript. AT carried out the chemical analysis. KN prepared the final draft of the manuscript, while MN and AT revised the manuscript critically. All authors read and approved the final manuscript.

## **Competing interest**

The authors declare that they have no competing interests.



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