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# ANTIMICROBIAL SUSCEPTIBILITY IN CLINICAL MASTITIS CASES ON SIMMENTAL FARMS IN THE MAČVA REGION (SERBIA)

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***SUMMARY***

*The battle against mastitis remains an enduring challenge in contemporary animal farming. This paper presents the frequency of bacterial pathogens isolated from milk samples collected on 3 farms with a previous history of clinical mastitis in the Mačva region during the year 2022. A total of 77 Simmental cows were enrolled in the study, ranging from the first to sixth lactation and managed in a tie-stall system. The results obtained indicate that the frequency of clinical mastitis in the animals considered was 16.9%. Of a total of 13 pathogenic isolates with clinically present mastitis, Staphylococcus aureus was recorded in 5 (38.4%), Streptococcus uberis in 3 (23.1%), Escherichia coli in 2 (15.4%), Klebsiella pneumoniae in 2 (15.4%), and Streptococcus dysagalactiae in 1 (7.7%). Notably, the largest number of clinical mastitis cases (namely 8, or 61.5%) was recorded during the winter period. Both cases of mastitis caused by K. pneumoniae were detected in the summer, the occurrence of which was implicated in changes in the bedding straw. According to the stage of lactation, the highest frequency of mastitis was found in the early stage of lactation totaling 8 cases (61.5%), followed by 4 (30.8%) in the middle phase of lactation, and only 1 (7.7%) in the late stage of lactation. The causative agents of the Streptococcus genus showed susceptibility to β lactamates in all the cases, whereas all of the E. coli and K. pneumoniae isolates were found resistant to ampicillin, amoxicillin with clavulanic acid.*

***Key words:*** *antimicrobial resistance, clinical mastitis, cows, frequency, Serbia*

## INTRODUCTION

The occurrence of clinical mastitis is common on high-milk cow farms, leading to economic losses due to milk rejection, therapy costs, and the elimination of cows from production (Seegers et al., 2003). The most common causes of mastitis in cows are bacteria from the genera *Staphylococcus*, *Streptococcus*, and coliform bacteria. The bacterial agents *S. aureus*, *S. uberis*, *S. dysagalactiae*, *Klebsiella pneumoniae* and *E. coli* are among the most frequently confirmed in Serbian dairy farms (Rogožarski et al., 2012; Zdravković et al., 2021; Ninković et al., 2023). A number of factors consequently cause the appearance of clinical mastitis, such as the stage of lactation, high milk production, general health status, hygiene condition, bedding quality, type of housing, flooring, holding conditions, milking procedures, udder cleanliness, and the previous history of mastitis (Elbers et al., 1998; Fesseha et al., 2021). Seegers et al. (2003) also showed that the occurrence of mastitis in cows is associated with high milk production, lactation stage, hygiene conditions, holding conditions, and the presence of stressors. Mastitis causes changes in the chemical composition of milk which makes it unsuitable for the production of dairy products (Muhee et al., 2017), resulting in financial loss. The process of preparing the udders for milking, and the milkers themselves, often result in the causative agent of mastitis spreading within the herd. The most common way to spread the causative agent of mastitis within herds is through milking equipment, using the same towels to wipe the udder (Elbers et al., 1998). Strengthening the prevention of mastitis and early detection can significantly reduce the need for antibiotics, thereby mitigating the further growth of antimicrobial resistance. There is no information available on the incidence of clinical mastitis in Serbia. It has been hypothesized that mastitis is present on Serbian farms, most frequently in the early stage of lactation and in all seasons. The following objectives were established in this study: 1) monitoring the presence of clinical mastitis on smallholder Simmental dairy farms, 2) identifying the causative agent of mastitis and determining the antimicrobial susceptibility of the isolates obtained, and 3) comparing the occurrence of clinical mastitis during various stages of lactation in all seasons.

## MATERIAL AND METHODS

### Sample collection

We studied the frequency of bacterial pathogens isolated from routine seasonal check milk samples on three farms with a previous history of clinical mastitis in the Mačva district, Serbia, in 2022. The data obtained are presented according to the stage of lactation and seasonal distribution. A total of 77 Simmental cows, aged 27 to 85 months, were enrolled in the study, ranging from the first to sixth lactation and managed in a tie-stall system with straw bedding. The observation period stretched from January to December 2022, during which all the cows received similar nutrition. All the herds had a concrete stall base with deep litter straw beds. The temperature range during this period varied between -5 ℃ and 38 ℃. Regular quarterly California mastitis tests were performed on all individual quarters as a screening test. Along with positive bacterial culture, clinical mastitis was diagnosed through clinical examination of the udder, which included assessing changes in milk quantity, appearance, the presence of clots, and a positive California mastitis test. Milk samples for mastitis testing were collected from all the animals to identify the causative agent and administer antimicrobial therapy where applicable. Udders were prepared for aseptic sampling by dry washing and disinfection of the teat orifice.

### Identification of clinical mastitis pathogens

After collection, the milk samples were placed in ice-cooled containers and transported to the laboratory within no more than 4 h. Microbiological examination was performed using standard procedures (Quinn et al., 2011). Briefly, the milk samples were inoculated onto Columbia blood agar, MacConkey agar, and Sabouraud dextrose agar plates (all from Torlak, Serbia). The primary isolates underwent the Gram staining procedure (Biomereux, France), followed by catalase and oxidase reaction tests (HiMedia, India). The initial diagnoses based on growth characteristics and enzyme reactions were further examined by latex agglutination tests for *S. aureus* (Slidex Staph, Biomereux, France) and Streptococci (Slidex StreptoPlus, Biomereux, France). For latex agglutination reactive Streptococci and for Gram-negative bacteria, oxidase negative, catalase positive bacteria, further biochemical tests were conducted, using commercial kits for Gram-positive and enteric nonfermenter bacteria, respectively (BBL Cristal, Becton, Dickinson, and company USA or API system, Biomereux, France).

### Antimicrobial testing

Antibiograms were performed following the ISO 20776-1 (2006) (International Organization for Standardization, 2006) standard, using the disk diffusion method and antimicrobial test disks (BBL, USA) to determine susceptibility toward penicillin (1 IU), and other antibiotics (with disc content in µg) such as cefoxitin (30), amoxicillin with clavulanic acid (20/10), ampicillin (5), cephalexin (30), ceftriaxone (30), gentamicin (10), tetracycline (30), and trimethroprim+sulfametoxasole (23.75/1.25) (where applicable). Interpretative zones were estimated following the antimicrobial testing standard (CLSI, 2018).

## RESULTS AND DISCUSSION

Clinical mastitis was diagnosed in 13 out of 77 cows, representing 16.9% of the total population. Out of a total of 13 milk samples collected from cows with clinically present mastitis, the presence of *Staphylococcus aureus* was recorded in 5 samples (38.4%), *Streptococcus uberis* in 3 samples (23.1%), *Escherichia coli* in 2 samples (15.4%), *Klebsiella pneumoniae* in 2 samples (15.4%), and *Streptococcus dysagalactiae* in 1 sample (7.7%). During the observation period, no mixed infections were diagnosed. The overall frequency of bacterial agents and antimicrobial susceptibility is shown in Table 1. Antimicrobial susceptibility is provided solely for commonly used antibacterials.

The isolates of *S. aureus* (n = 5) revealed a high sensitivity to the β lactam antibiotic group (100%), whereas *K. pneumoniae* and *E. coli* showed the highest antibiotic resistance of all the isolates obtained.

Table 1. Frequency of bacterial agents and their antimicrobial sensitivities

| | ***S. aureus* n=5** | | ***S. uberis* n=3** | | ***S. dysgalactiae* n=1** | | ***E. coli* n=2** | | ***K. pneumoniae* n=2** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | S | R | S | R | S | R | S | R | S | R |
| Amoxicillin with clavulanic acid | 5 | 0 | 3 | 0 | 1 | 0 | 0 | 2 | 0 | 2 |
| Ampicillin | 5 | 0 | 3 | 0 | 1 | 0 | 0 | 2 | 0 | 2 |
| Cefalexin | 5 | 0 | 3 | 0 | 1 | 0 | 0 | 2 | 0 | 2 |
| Ceftriaxone | 5 | 0 | 3 | 0 | 1 | 0 | 2 | 0 | 2 | 0 |
| Gentamicin | 1 | 4 | 0 | 3 | 0 | 1 | 2 | 0 | 1 | 1 |
| Tetracycline | 3 | 2 | 1 | 2 | 1 | 0 | 1 | 1 | 2 | 0 |
| Trimetroprim+ Sulfamethoxazole | 3 | 2 | 0 | 3 | 0 | 1 | 2 | 0 | 1 | 1 |

Legend: S – susceptible; R - resistant

Seasonal dynamics revealed a predominance of winter mastitis cases 8, accounting for 61.5% of the recorded mastitis cases, whereas during the summer, the clinical form of mastitis was solely attributed to *K. pneumoniae* (Fig. 1). The clinical form of mastitis primarily manifested in the early stage of lactation (8 cases, or 61.5%), followed by the middle stage of lactation (4 cases, or 30.8%) cases, and only two cases (7.7%) of clinical mastitis occurring in the late lactation phase.

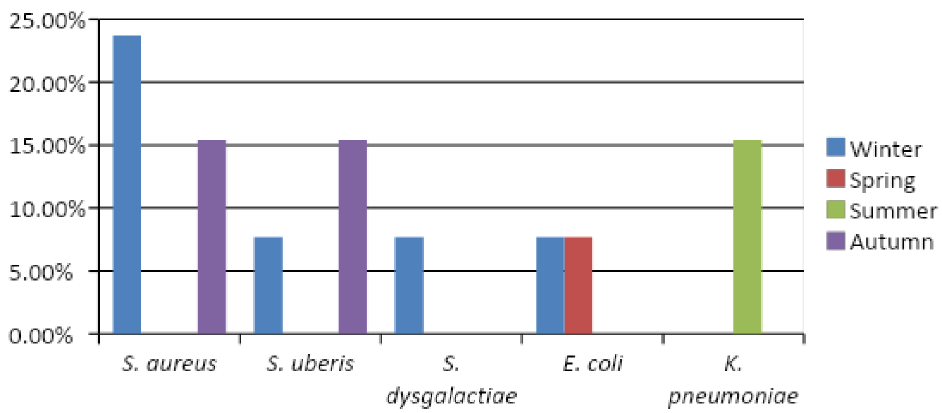


Figure 1. Distribution of the occurrence of mastitis and bacterial agents during seasons

The occurrence of clinical mastitis on farms leads to significant economic losses, implicated in the decrease in milk production and the culling of cows from production. Intensive milk production and the frequent use of antimicrobial drugs increase the occurrence of multi-drug resistance. The presence of *S. aureus* was confirmed in 20% of the subclinical mastitis cases in Vojvodina, Serbia (Savić Radovanović et al., 2017). The bacteria *S. uberis* and *S. aureus* are also dominant mastitis pathogens worldwide, including in New Zealand (McDougall et al., 2014). However, *E. coli* was the most often isolated from cases of clinical mastitis in Greece, accounting for 49.3% of the cases studied (Zdragas et al., 2017). The study by Idriss et al. (2014) found the frequencies of bacterial agents *E. coli*, *S. aureus* and *S. uberis* to be 12.82%, 9.74% and 4.10%, respectively. Multiple resistance to five and more antibiotics was found in *S. uberis*, except for ampicillin, amoxicillin, and ceftriaxone, which was previously identified by McDougall et al. (2014). Our results are consistent with those of Zigo et al. (2018) indicating the susceptibility of *S. aureus* and *S. uberis* isolates to penicillins such are amoxicillin with clavulanic acid and ampicillin. The greatest variation in antimicrobial susceptibility to drugs was present in the *S. aureus* isolates. Our isolates of *S. dysagalactiae* exhibited the highest susceptibility to β-lactam antibiotics, which is consistent with the results of a number of authors (Guérin-Faublée et al., 2002; Cojkić et al., 2015). Although Coliform bacteria such as *E. coli* and *K. pneumoniae* demonstrate similar antimicrobial susceptibilities, the importance of a precise differential diagnosis between them is still underscored. Our findings align with those of Munoz et al. (2007) regarding the occurrence of mastitis caused by *K. pneumoniae* during the summer. Osterås et al. (2006) reported mastitis caused by *E. coli*, *S. dysagalactiae*, and *S. aureus* as the most prevalent in winter. Our results are consistent with the reports mentioned above, confirming that the most frequently isolated bacteria were *S. aureus* and *S. uberis*, and that the occurrence frequency of clinical mastitis was the highest in the early stage of lactation (51.4%) (Shpigel et al., 1998). Despite other publications presenting results contrary to ours, such as the study by Belayneh et al. (2013) reporting the highest prevalence of mastitis in the late stage of lactation, our findings indicate that the early lactation period of cows is also affected by hormonal changes, resulting in frequent mastitis (Vangroenweghe et al., 2005). Moosavi et al. (2014) found that winter-spring mastitis tends to occur in the early stage of lactation, whereas summer clinical mastitis is associated with the late stage of lactation. Factors linked to seasonal variations, such as hygiene conditions in facilities and the cleanliness of milking equipment, are significant contributors to the occurrence of mastitis on farms, as highlighted by Osterås et al. (2006).

## CONCLUSION

The frequency of clinical mastitis in the dairy Simmental cows under observation was 16.9%, with the presumptive expected frequency of clinical mastitis being higher in the early stage of lactation and during the winter season. Further studies aimed at expanding these observations would offer a comprehensive understanding of the frequency of clinical mastitis on Serbian dairy farms and the extent of antimicrobial resistance at the national level.

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***Conflict of interest:*** The authors declare that they have no conflict of interest.

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