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Prevalence and etiology of mastitis in dairy herds in eastern Slovakia

Abstract

Despite continuous advancements in the farm management of primary milk production, inflammation of the mammary gland - mastitis remains a critical challenge in dairy herds, adversely affecting milk quality and food safety. The primary objective was to evaluate the impact of environmental and physiological factors on mammary gland health by identifying intramammary infections (IMI) and the associated pathogen spectrum in dairy cows during the first 100 days of lactation. A study conducted in 10 herds in eastern Slovakia on a sample of 1,000 dairy cows (100 cows from each farm) during early lactation confirmed that mammary gland health is fundamentally threatened by intramammary infections (IMI) and a specific spectrum of pathogens. The findings revealed a 20.7% overall incidence of mastitis within the monitored herds. Subclinical mastitis was the most common, affecting 14.2% of the examined cows, while clinical mastitis (CM) accounted for 6.5% of the positive cases. Microbiological analysis identified staphylococci as the dominant etiological agents, responsible for 55.8% of IMI cases. Other significant pathogens included Gram-negative bacteria (18.6%) and streptococci (17.8%). Beyond causal pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae*, coagulase-negative staphylococci are increasingly prominent. Due to their high pathogenicity and virulence factors including biofilm formation, hemolysin production, and DNA hydrolysis play a pivotal role in the pathogenesis of IMI. Targeted management of these emerging pathogens is essential to minimize economic losses for producers and ensure public health protection against milk-borne diseases.

Keywords: early lactation, subclinical mastitis, pathogens, coagulase-negative *staphylococci*

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Introduction

Milk production is a key aspect of animal farming, with 150 million farmers supplying over 6 billion consumers. Although dairy products have a unique composition, EU laws strictly require that raw milk used for production meets all nutritional and hygiene standards; therefore, monitoring the health of production animals is crucial for all farmers involved.[1]

Despite ongoing improvements in zoohygienic standards on farms, mastitis remains one of the most significant health challenges. It is a complex inflammatory response of the body, primarily aimed at eliminating invading microorganisms and then restoring damaged udder tissue, which is essential for continued milk production. The severity and progression of this disease vary among individuals, depending on the tissue's reaction to the infection.[2] Subclinical mastitis is the most prevalent in cattle, causing a 70% reduction in milk yield and a significant increase in somatic cell count (SCC). Clinical mastitis is characterized by an inflamed, discolored udder and changes in milk, including blood, pus, flakes, and clots.[3] The economic consequences of mastitis include reduced milk production, poor quality milk, increased culling rate and increased cost of veterinary services and medicine. Clinical or subclinical mastitis in cattle are caused by microorganisms, mostly bacteria, including *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae*, *Escherichia coli, Enterococcus* spp., and *Pseudomonas* spp., fungi such as *Candida* spp. or *Cryptococcus* spp., and algae, e.g., Prototheca. Bacteria are always shedding in milk, posing a potential threat to humans by causing milk-borne diseases.[4,5]

The main causes of mastitis are poor udder and milking hygiene and environmental conditions of cows. However, microbial contamination of milk can originate from the animal itself or occur along the milk supply chain. Additionally, recontamination may occur after milk processing due to unhygienic conditions, improper handling, or improper storage of milk during consumption.[6] Therefore, routine monitoring of the microbiological status of milk throughout the supply chain is essential to protect public health and reduce losses from milk condemnations.[7,8] The objective of this study was to estimate mastitis prevalence and assess bacterial contamination levels in milk from 10 dairy herds in eastern Slovakia.

Material and methods

Monitored dairy farms

The practical part of the study was carried out on 10 dairy farms in eastern Slovakia, specifically in the Prešov and Košice regions. The farms were chosen to use conventional Slovak piebald cattle and their crossbreds, producing milk yields of 6.8–8.3 kg x 10^3 during the 2nd to 5th lactations. Dairy cows on all monitored farms were kept in a free-range system on straw bedding, with *ad libutum* access to water and fed total mixed ratio composed of silage, hay, and concentrate, meeting the nutritional needs[9] of dairy cows weighing 650 kg and producing an average of 15-25 L of milk daily. All cows were milked twice a day in either a parallel (BouMatic, USA) or dovetail (DeLaval, Sweden) milking parlor, with pre- and post-dipping solutions applied.

Examination of dairy cows

Dairy cows from the studied farms were selected to form production groups based on lactation stage and nutritional phase, as determined by zootechnicians. The sample size of 100 lactating cows was estimated using Kothari's formula.[10] The selected cows were examined for both clinical and subclinical mastitis. From each farm, 100 cows were examined and grouped according to calving and lactation period (14-100 days after calving). Clinical mastitis examination involved visual inspection and palpation of the udder for abnormalities like swelling, hardness, heat, redness, or pain, as well as lesions on the teats. Stripping teats to get a few squirts of milk into a strip cup, which was enhanced for checking abnormalities such as watery appearance, clotted or blood-stained milk, color changes, clots, and flakes. Screening for subclinical mastitis was conducted using the California Mastitis Test (CMT). Briefly, milk was collected from each quarter into the corresponding CMT white plastic paddle, with approximately 2 mL in each container (Figure 1, Table 1).

Table 1 Evaluation of Califonia Mastitis test scores and equivalent somatic cell counts

CMT test score	Reaction observed	Equivalent milk SCC/ ml x 103	Health quarter	No. of tested quarters	% of tested quarters
Negative	Homogenous mixture, no thickening	0-200, 0-25% Neutrophils	Health quarter	3,513	87.8
Trace	Slight (a faint cloud like) thickening that disappear about 10 minutes	150-500, 30-40% Neutrophils	Possible infection if all quarters read trace, there is infection. If one or two quarters read trace, infection is possible.	41	1
1	Distinct slime formation occurs immediately after mixing but there is no tendency to form gel, this slime does not disappear over time	400-600, 30-60% Neutrophils	Subclinical mastitis	233	5.8
2	Distinct slime formation occurs immediately after mixing with a slight gel formation	500-800, 60-70% Neutrophils	Subclinical mastitis	101	2.5
3	Distinct slime formation occurs immediately after mixing; a distinct gel forms, and the mixture's surface becomes elevated.	>800 000, 70-80% Neutrophils	Subclinical/clinical mastitis	112	2.8

Figure 1 Sensory evaluation of milk in a double-bottomed cup and assessment of CMT.

The CMT reagent (Shalma test reagent R) was dispensed without frothing from a CMT reagent dispenser until an amount equal to the volume of milk in each test cup was reached. Mixing was performed by gently swirling the paddle in a horizontal circular motion. The reaction reached its peak color within 10 seconds. Each sample test was then graded, interpreted, and scored into four categories according to Tančin et al.[11]

For laboratory diagnostics of bacterial pathogens, according to Holko et al.[12] mixed samples of raw milk were collected from 228 cows with CMT scores ranging from trace to 1-3. Based on mastitis classification,[13] individual cases were evaluated as subclinical or clinical IMI (Figure 2), using clinical examination of the udder, increased SCC according to CMT assessment, and laboratory analysis of the collected milk samples. Clinical mastitis (CM) was categorized as mild (CM1), characterized by visible changes in secretion; moderate (CM2), which also showed local signs of inflammation in the mammary gland; and severe (CM3), which additionally exhibited general signs such as fever, low temperature, loss of appetite, or inability to stand.

Figure 2 Clinical mastitis with change in milk secretion consistency caused by *S. aureus* confirmed by primary culture on blood agar.

Sample cultivation and detection of udder pathogens

Milk samples were thawed at room temperature. An inoculum of 0.2 ml from each sample was plated on 5% blood agar plates, incubated at 37 °C, and evaluated after 24 hours. Based on morphological features, the initial colonies grown on blood agar were re-cultured onto various selective media[14] for an additional 24 hours at 37 °C. Identification of *Staphylococcus* spp. involved assessing growth on selective agars (5% blood agar, agar 110, Baird-Parker agar, Brilliance UTI Clarity Agar, OXOID Ltd, UK). Colonies were then identified by their shape, Gram stain, hemolysis type, catalase activity (3% H_2O_2, Merck, Darmstadt, Germany), esculin hydrolysis, and cytochrome C oxidase (Bactident Oxidase, Merck). Esculin-positive streptococci were cultured on modified Rambach agar for more precise identification of *Str. uberis* or *Enterococcus* spp., following the studies of Vasil' et al.[14] and Holko et al.[12]

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Identification of each species using biochemical tests, specifically STAPHYtest 24, STREPTOtest 24, or ENTEROtest 24, and evaluated the results with the TNW ProAuto 7.0 software (Erba-Lachema, Brno, CZ), achieving over 90% accuracy in species identification. Additionally, for all gram-negative species, MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) was employed for detection via laser desorption ionization mass spectrometry.[15] To verify these identifications, reference strains *S. aureus* CCM 7113 and *Streptococcus uberis* CCM 4617 (CCM, Masaryk University, Brno, CZ) were used as controls.

Culture samples were considered positive if one or more colony-forming units of main udder pathogens, such as *S. aureus, Str. dysgalactiae*, or *Str. agalactiae*, were detected. A sample was also considered positive if growth of a contagious udder pathogen was confirmed along with other environmental pathogens, or if these pathogens produced at least three CFU. If no major contagious pathogens were found or if three or more pathogens were isolated from a single milk sample, such cultures were deemed contaminated.

Detection of virulence factors in *staphylococci*

Confirmed staphylococci by MALDI-TOF analysis were tested for extracellular proteolytic enzyme production (gelatin hydrolysis assay) and deoxyribonuclease assay (DNase assay) according to a previous study by Hiko.[16] Biofilm formation was determined by the phenotypic method on Congo Red agar (CRA) according to Vasil' et al.[17] The ability of staphylococci to produce hemolysins was determined as described by Moraveja et al.[18] The types of hemolysis were phenotypically characterized based on the lysis zone of each staphylococcal isolate on blood agar plates supplemented with 5% sheep blood after 24 and 48 hours of incubation at 37 °C.

Data analysis

Microsoft Excel 2007® (Microsoft Corp., Redmond, USA) was used to process the obtained results, and SPSS version 20 and Excel (IBM Corp., Armonk, USA) were used for analysis. Based on the specific microbial species and forms of mastitis, the cultured pathogen findings were processed and converted to percentages. According to the production of virulence factors, staphylococcal isolates were compared using Chi-square and Fisher's exact test at a critical probability of $P<0.05$ at 95% confidence interval. The significance level $\alpha = 0.05$ between the compared groups of staphylococci was set at a critical value $\chi 2 = 2.803$. Testing value (G) and statistical independence of virulence factors in isolated staphylococci was confirmed when $G > \chi^2$; the independence was not statistically significant when testing value was $G < \chi^2$.

Results and discussion

Currently, mastitis is a major health issue in dairy cows, closely linked to their overall health and specific lactation stages.[19] The study focused on tracking the prevalence and causes of mammary gland inflammation across 10 farms in eastern Slovakia, especially during the critical early lactation phase. Cows included in the study were between the 14th and 100th days after calving, which is the most important period on farms, as they produce about 42-45% of the total milk during this period. The high production demands in the first 100 days put cows under intense stress. This stress stems from hormonal changes related to lactogenesis, decreased dry matter intake, and the need for high milk yield, which results in a negative energy balance. These factors cause significant lipomobilization of body fat and changes in body condition, weakening the immune system and increasing vulnerability to mammary gland infections.[20]

The study results show that the incidence of mastitis in the monitored herds during early lactation was 20.7% (Figure 3). Subclinical mastitis accounted for the largest proportion at 14.2%, with ranges of 6.3% to 22.1% among the examined cows in monitored farms. Clinical mastitis made up 6.5% of the total number of positive cows, with intervals from 2.5% to 10.6% in the monitored farms. According to Tvarožková et al.,[21] the subclinical form of mastitis is considered 15-40 times more common than the clinical form and leads to greater losses in milk production, also serving as a reservoir of infectious organisms. Subclinical mastitis is associated with various factors, including breeding practices, milk hygiene, seasons, and cows' ages. The current study was conducted during early lactation (the first 100 days after calving), which may be associated with a higher incidence of clinical mastitis in dairy cows due to environmental and physiological factors. These risk factors negatively affect both specific and non-specific immunity in dairy cows, making it easier for environmental pathogens to enter the mammary gland.[20]

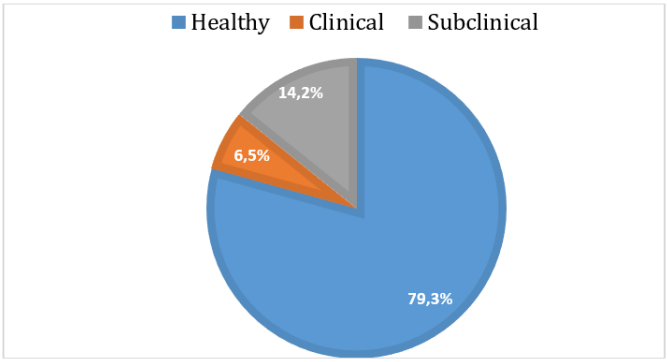

Figure 3 Occurrence of mastitis in monitored dairy farms.

Note: Subclinical mastitis - characterized by the absence of clinical symptoms; the mammary gland and milk appear normal without visible changes, yet infection persists, as indicated by a positive CMT-score and an elevated SCC. Clinical mastitis: exhibits observable symptoms, which can be classified as mild, moderate, or severe.

The key indicator of IMI onset is an increased somatic cell count (SCC) confirmed by CMT analysis. CMT is one of the fastest methods for assessing SCC levels on farms and, together with milk yield monitoring, provides the farmer with relevant information about mammary gland health.[11] Out of 1000 examined dairy cows, 772 (77.2%) tested negative with the CMT, while 228 (22.8%) showed a positive reaction with CMT score 1-3. When evaluating the CMT score, 487 (12.2%) quarters had trace or positive results in the 1-3 range (Table 1). Based on the history, clinical examination, and positive CMT, 215 (74.7%) isolates causing subclinical or clinical mastitis were cultured from the 228 mixed samples, posing a serious risk to individual animal health and the potential spread of infection within the herd.

The results of the culture and identification of mammary gland pathogens are presented in Table 2. Of the 215 samples positive for bacterial mammary gland pathogens, 120 (55.8%) were confirmed as staphylococci, with CNS (40.0%) being the most frequently detected. Among the staphylococci, the second most common and pathogenic isolate was *S. aureus* (15.8%), which primarily caused clinical mastitis. Similarly, Gram-negative pathogens were isolated from mastitic samples (Table 2), indicating fecal contamination and an urgent need to address environmental mastitis. According to a study by Xu et al.,[22] some strains of *E. coli* are verocytotoxigenic, such as enterohemorrhagic *E. coli* O157:H7, which cause hemorrhagic colitis.

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Table 2 Udder pathogens isolated from milk samples and their contribution to individual forms of mastitis

Pathogen	No. of isolates	% (n=215)	Clinical mastitis						Subclinical n/%	
			CM1		CM2		CM3			
Staphylococci			n	%	n	%	n	%	n	%
CNS	86	40	9	4.2	3	1.4	4	1.9	70	32.6
Staph. aureus	34	15.8	11	5.1	4	1.9	7	3.3	12	5.6
Streptococci										
Str. uberis	19	8.8	4	1.9	4	1.9	2	0.9	9	4.2
Str. agalactiae	6	2.8	3	1.4	0	0	0	0	3	1.4
Streptococcus spp.	13	6	2	0.9	1	0.5	0	0	10	4.7
Others										
Enterococcus spp.	17	7.9	2	0.9	0	0	0	0	15	7
Escherichia coli	23	10.7	1	0.5	1	0.5	4	1.9	17	7.9
Mixed infection[4]	17	7.9	5	2.3	1	0.5	0	0	12	5.6
Total	**215**	**100**	**37**	**17.2**	**14**	**6.5**	**17**	**7.9**	**148**	**68.8**

Note: CNS – coagulase-negative *staphylococci*; CM[1-3] - clinical intramammary infection (IMI), including mild (CM1), moderate (CM2) and severe forms (CM3) of mastitis; Subclinical- subclinical intramammary infection. Mixed infection4 – include a mix infection of two bacterial pathogens.

Interestingly, CNS and *S. aureus* represented the majority of isolated bacteria in milk, especially in dairy cows. However, other common bacteria were *Str. uberis*, *Str. galactiae* and *E. coli*. Bacteria are the main cause of infectious mastitis, both clinical and subclinical, in dairy cows. These results suggest that isolated bacteria should be considered when planning mastitis control in monitored herds. Other studies have observed similar results in Slovakia,[12,21] the Czech Republic,[23] Poland,[5,6] and elsewhere, indicating a common occurrence of mastitis pathogens. In addition, bacteria can be present in the teat canal or on the udder surface, as is usual, without an inflammatory reaction.[19] Consumption of milk contaminated with *S. aureus* may pose a health risk, as approximately 10% of mastitic staphylococci produce heat-stable enterotoxins.[24]

Some studies have linked *S. aureus* to gastroenteritis through these enterotoxins.[18,24,25] Since *S. aureus* is contagious and commonly colonizes the teat tips and teat canal, therapies such as dry teat disinfectants and post-milking teat treatments can be very valuable in controlling mastitis in lactating cows caused by *S. aureus*.[7] However, these measures are not always effective, as up to 67.6% of *S. aureus* and 39.0% of CNS have been shown to form biofilm (Table 3), which is a key virulence factor that protects them from disinfectants.

Table 3 Virulence factors in *staphylococci*

***Staphylococcus* spp. / no.**	**Hemolysins[1]**	**DNAse[2]**	**Gelatinase**	**Biofilm**	**Testing value**
S. aureus (34)	12 α /6 δ /4 β	26	32	23	5.225*
Coagulase-negative *staphylococci* with a significant presence of virulence factors					
S. warneri (23)	9 β /5 δ	7	7	9	3.314*
S. chromogenes (20)	5 δ /8 β	5	4	8	2.975*
S. xylosus (16)	3 δ /6 β	4	1	6	2.833*
Coagulase-negative *staphylococci* with low levels of virulence factors					
S. epidermidis (11)	3 δ	0	0	3	1.412
S. haemolyticus (8)	2 β /1 δ	1	0	2	0.905
S. capitis (4)	1 δ	0	0	0	0.341
S. piscifermentans (2)	1 β	0	0	0	0.28
S. hyicus (2)	0	0	0	0	0.001

Note: hemolysines[1] - production of hemolysin type α, β or δ; DNAse[2] - ability of *staphylococci* to hydrolyze DNA; *Chi-squared test significance level α = 0.05; critical value χ 2 = 2.803; Testing value (G) and statistical independence of virulence factors in isolated *staphylococci* was confirmed when G > χ 2; the independence was not statistically significant when testing value was G < χ 2.

The high occurrence of IMI caused by staphylococci is due to their considerable pathogenicity and ability to produce multiple virulence factors, which play a crucial role in the development of new infections and hinder their treatment and removal from the environment.[7] Specifically, the species *S. aureus, S. chromogenes, S. warneri*, and *S. xylosus* isolated from mastitis milk demonstrated the broadest range of virulence factors ($p<0.05$), including biofilm formation, hemolysin production, and ability of staphylococci to hydrolyze DNA (Table 3).

In agreement with Perez et al.,[26] it can be inferred that the synergistic interaction between hemolysin production and biofilm formation significantly increases staphylococcal adherence to the mammary gland epithelium. This mechanism facilitates the survival of pathogens during the host immune response and, at the same time, reduces the penetration of disinfectants and antimicrobial agents. Moreover, in a study by Zigo et al.[20] on the etiology of mastitis in dairy farms, it was confirmed that CNS forming biofilms were multiresistant to more antimicrobial agents than isolates without this ability. Given the limited therapeutic efficacy of conventional antibiotics against biofilm, it is necessary to place greater emphasis in breeding practice on early diagnosis and targeted management of infections caused by these virulent strains.

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Conclusion

Based on the results, it can be concluded that a 20,7% prevalence of mastitis during early lactation was observed among the 10 monitored dairy farms in Eastern Slovakia. Subclinical mastitis (14.2%) was the most common form of IMI; however, an increased occurrence of clinical cases (6.5%) was observed during early lactation, which negatively affects health and milk production. Most IMIs were caused by staphylococci in 55.8% of positive cases, specifically CNS and *S. aureus*. In addition to *S. aureus*, some CNS (*S. warneri, S. chromogenes*, and *S. xylosus*) may exhibit the same degree of pathogenicity due to the presence of multiple virulence factors, including biofilm production, gelatinase activity, hemolysis, and DNA hydrolysis. Consequently, in farming practice, it is essential to place greater emphasis on early diagnosis and targeted management of infections caused by these virulent strains.

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Conflicts of interest

The Authors declares that there are no conflicts of interest.

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