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Original Scientific Article

PREVALENCE OF SUBCLINICAL MASTITIS PATHOGENS IN SMALL DAIRY FARMS IN REPUBLIC OF NORTH MACEDONIA

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ABSTRACT

Subclinical mastitis is an asymptomatic udder infection distributed worldwide with enormous losses in the dairy industry. The study's objective was to determine the presence of this pathological condition in small dairy farms in the R. of N. Macedonia and to identify the most common associated bacteria. Milk samples were obtained from 96 dairy cows (378 udder quarters) in seven dairy farms, in 3 consecutive samplings 24-72 hours apart. The samples were cultured on routine bacteriological growth media and incubated for 24-48 hours. The isolates were identified by AximaiD Plus MALDI-TOF MS Platform. Subclinical mastitis was found in 49 animals (51%) and 104 infected quarters (27%). The most frequent isolated bacteria on cow level were *Streptococcus uberis* (19.4%), *Staphylococcus haemolyticus* (13.4%), *Staphylococcus aureus* (7.4%) and *Staphylococcu ssimulans* (7.4%). On quarter level, the most isolated pathogen was *Streptococcus uberis* (35.6%) followed by *Staphylococcu shaemolyticus* and *Staphylococcus aureus* (10.3% and 9.2% respectively). Subclinical mastitis was found to be highly present in the selected small dairy farms. The most prevalent bacteria identified in the dairy farms (*Streptococcus uberis*, *Staphylococcus aureus* and coagulase–negative staphylococci) indicate that poor management and udder health practices, inadequate milking procedures and lack of mastitis control strategies greatly contribute to occurrence and persistence of subclinical mastitis.

Key words: subclinical mastitis, MALDI-TOF, bacteria, milk

INTRODUCTION

Mastitis is an inflammation of the mammary gland (MG) characterized with physico-chemical and microbiological changes in the milk quality, increased somatic cell count (SCC) and pathologically changed mammary tissue (1). The subclinical mastitis (SM), defined as a non-symptomatic intramammary inflammation (2), is the most common form of mastitis, 15 to 40 times more prevalent than the clinical mastitis (4). It affects 20-50% of cows in given herds (3), with great economical losses, affecting the dairy herds worldwide (5, 6). The economic losses due to SM are: decreased milk production, discarding abnormal milk and milk from cows treated with antibiotics, low milk quality and price due to high somatic cell count (SCC) and bacteria in milk, medication costs, costs for veterinary services, increased risk of developing clinical mastitis (CM), herd replacement, antibiotic residues in milk and milk products etc (7). Moreover, cows with SM are reservoirs for infection, increasing the risk for spreading the infection in the healthy cows within and between herds which emphases the importance of this condition on a national level (5, 8).

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The intramammary infection (IMI) can be triggered by more than 135 microorganisms (9,10). The most prevalent ones can be grouped as (i) contagious/highly pathogenic bacteria: *Streptococcus agalactiae* and *Staphylococcus aureus*, and (ii) environmental bacteria: coagulase-negative staphylococci (CoNS), *Escherichia coli*, *Corynebacterium bovis*, *Streptococococcusuberis* and *Streptococcus dysgalactiae* (6, 11, 12).

The average SCC from healthy/non infected udder is around 50 000 cells/ml, and in most cases the value is bellow 150 000 cells/ml (13). The elevated SCC is the most frequently used indicator and internationally recognized standard for determination of subclinical mastitis (11, 14, 15). The SCC can be influenced by: mastitis-related factors (microorganisms, toxinsand mammary tissue damage), physiological factors (lactation stage, race), pharmacological factors (medications) as well as stress (diet change, transport, holding conditions, method of milking, milking machine etc.) (13, 16). However, the greatest increase in SCC is due to mastitis (12, 13, 14) as a result of primary immunological response and entry of polymorphonuclear cells in the milk cistern (11, 13).

The SCC can be performed on udder level - composite milk (from all quarterstogether) and quarter level – from each quarter separately. If the SCC is performed on composite milk, the increase in SCC in one quarter can be overlooked due to the dilution effect of the milk from the other healthy quarters (17). This fact was supported by the finding of Berglund et al. (18), where the cow composite milk samples with a low SCC (<100 000 cells/ml) were hiding more than 10% of individual quarters with elevated SCC and more than 50% of those quarters were infected. This highlights the importance of detecting the IMI on quarter level prevalence. On the other hand, the determination of SCC on cow level is important for individual udder health control, breeding and management purposes (18).

According to our national requirements for hygiene rules for food of animal origin (19) which are aligned with the EU regulation EC 853/2004 (20), the raw milk can contain up to 400 000 SCC and 100 000 colony forming units (cfu)/ml.

The studies on SM in the Republic of North Macedonia in terms of prevalence of SM, determination of the causative agents, their prevalence and average SCC are limited. The objective of this study was to determine the SM prevalence in the corresponding dairy farms as cow-level prevalence and quarter–level prevalence, to determine the causative agents of SM and to determine the average value of the SCC in SM positive and SM negative cows.

MATERIAL AND METHODS

Dairy farms and sample collection

The study was carried out in 7 randomly chosen dairy farms in different locations in the Republic of North Macedonia, and the samples were collected in the period of September - December 2018. The average number of cows per farm was 16, with 9 in the smallest and 30 cows in the largest one. All of the farms had similar husbandry practices i.e. the cows are kept and milked indoors in tie-stall system using straw as bedding, with twice-a day milking, poor housing hygiene, poor cow cleanliness and not using disinfection prior to and/or after milking. The number of tested cows and quarters is shown in Table 1.

Table 1. Number of tested cows and udder quarters

Farm	Lactating cows	Dry dairy cows	Cows under antibiotic treatment	Total	Udder quarters
A	27	1	2	30	106
B	7	3	-	10	26
C	12	2	2	16	48
D	12	1	-	13	47
E	19	3	1	23	76
F	10	2	-	12	40
G	9	-	-	9	36
Total	**96**	**14**	**3**	**113**	**378**

The milk samples were sampled from every (non-affected and dried) quarter for SCC and bacteriology. Every quarter was sampled three times, in a time frame of 48-72 hours. The samples were collected aseptically according to the recommendations from the National mastitis council (21) and properly marked with ear tag number of the sampled cow and udder quarter (front-left/FL, front-right/FR, rear-left/RL and rear-right/RR). The milk samples for SCC were sampled in sterile plastic tubes containing Broad Spectrum MIcrotabs® II (Advanced instruments) as preservative. As cows with blind (dried) quarters were included, those cows contributed with less than 8 samples per cow.

After collecting, the samples were transported cooled on 4°C in the laboratories of the Faculty of Veterinary Medicine in Skopje (FVMS) for further analysis.

Microbiological analysis and SCC

The microbiological analysis was performed in the Laboratory for microbiology in FVMS. Three hundred seventy eight (n=378) milk samples (0,01 ml) were plated on blood agar (Oxoid) with 5% sheep blood and incubated on 37°C for 24-48 hours (10, 21, 22). After the plate inspection, the criteria for positive udder quarter declaration were: two positive out of three consecutive milk samples and >1cfu for *Staphylococcus aureus* and *Streptococcus agalactiae,* and >10cfu for other microorganisms. The criterion for SM positive cow was the bacteria's presence in the sample, regardless which bacteria was isolated (Staphylococci, Streptococci etc) and the presence of one (or more) infected quarters. Samples yielding >3 bacterial species were considered contaminated (5).

After the initial isolation, the suspect colonies were subcultured to obtain pure cultures, and the isolates were then identified using MALDI-TOF MS Axima™ Confidence spectrometer (Shimadzu-Biotech Corp., Kyoto, Japan) in positive linear mode (m/z=2,000–20,000). This analysis was performed in the Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov" - Macedonian Academy of Sciences and Arts in Skopje. In short, a small amount of each pure culture was transferred to a FlexiMass™ target well using a disposable loop, overlaid with 1.0 µl of α-Cyano-4-hydroxycinnamic acid matrixsolution (CHCA; 40 mg/ml in water/acetonitrile/ethanol (1:1:1) with 0.03% trifluoroaceticacid and air-dehydrated within 1–2 min at 24–27°C (23). The reference strain *Escherichia coli* DH5α was used as a standard for calibration and as reference for quality control. Protein mass profiles were obtained with detection in the linear positive mode at a laser frequency of 50 Hz and within a mass range from 2.000–20.000 Da. A minimum of 50 laser shots per sample was used to generate each ion spectrum. Spectra were analyzed using SARAMIS™ (Spectral Archive And Microbial Identification System, AnagnosTec GmbH, Potsdam, Germany), a software in which the identification at the species level is based on a percentage of confidence referred to reference spectra (SuperSpectra™) that contain family, genus and species specific m/z biomarkers, as described in the SARAMIS™ user manual (24).

For the SCC, the samples were analyzed in the Laboratory for raw milk quality in FVMS, using the Fossomatic 6000 (Foss Electric, Denmark) according to ISO 13366-2:2006 (25).

RESULTS

The cow-level prevalence of SM in the corresponding dairy farms was 51% (49/96). The most prevalent bacteria in these cows were *Streptococcus uberis, Staphylococcus haemolyticus, Staphylococcus aureus* and *Staphylococcus simulans* (Fig. 1)

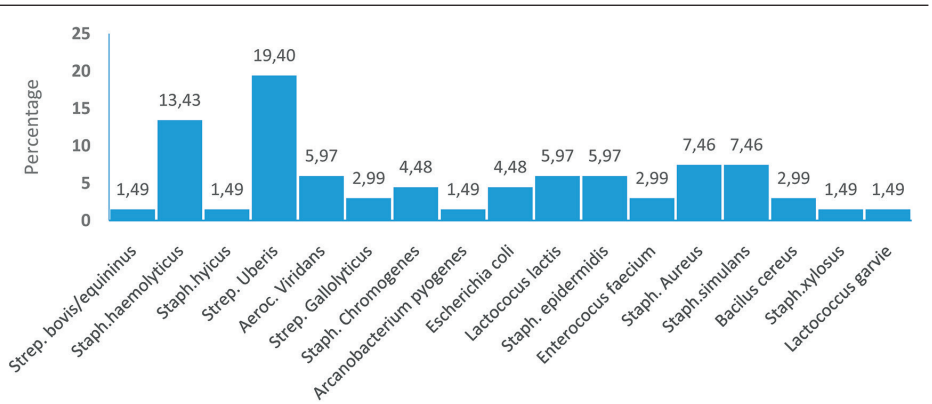

Figure 1. Percentage of isolated bacteria on cow level

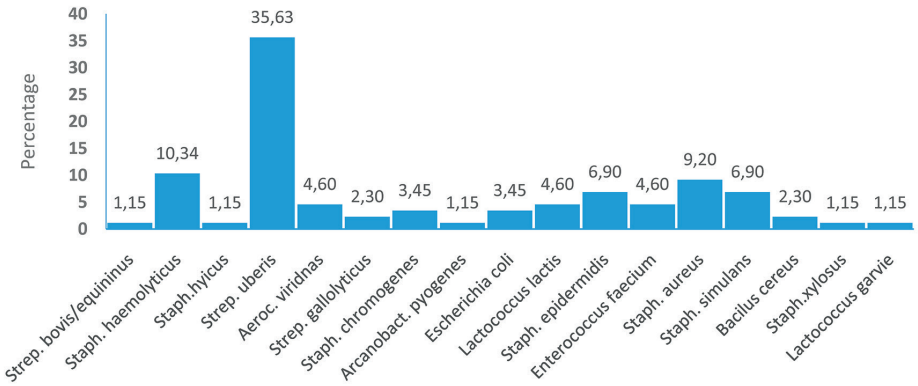

Figure 2. Percentage of isolated bacteria on quarter level

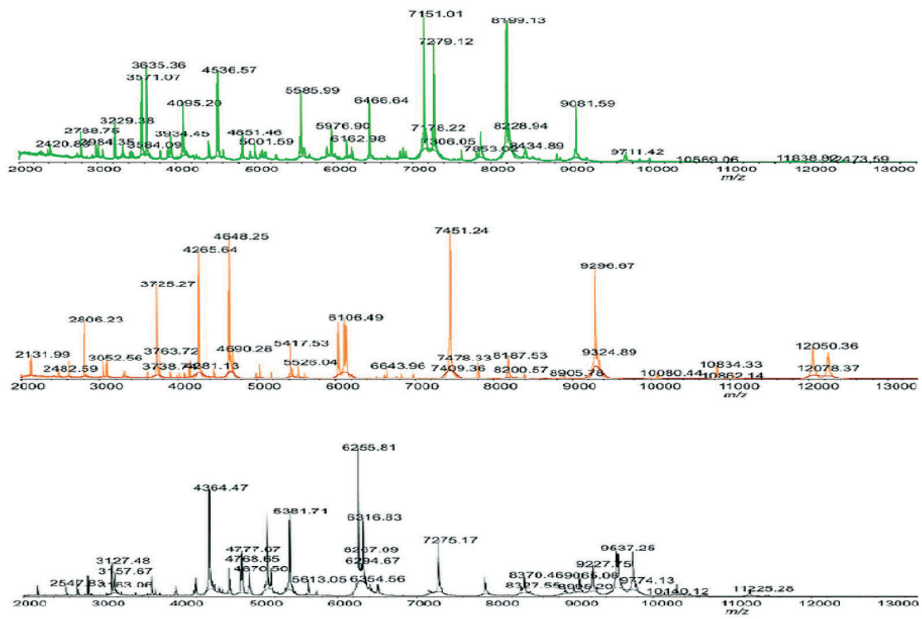

Figure 3. Representative spectrum of identified bacteria

The quarter level prevalence was 27% (104/378). The most prevalent bacteria on quarter level were *Streptococcus uberis, Staphylococcus haemolyticus* and *Staphylococcus aureus* (Fig. 2).

In total, eighty seven isolates were identified on species level using the spectral analysis of the SARAMIS™ database (Fig. 3). Most of the infections were only by one pathogen species, and mixed one with two pathogen species *(Staphylococcus haemolyticus and Aerococcus viridans)* was diagnosed in one quarter only.

The quarter – level prevalence for FL, FR, RL and RR was 27%, 23%, 22% and 28% respectively. Therefore, statistical significance was not determined between the infected quarter and quarter-level prevalence ($\chi^2(3,N=104) = 1.55$, $p > 0.05$).

Regarding the SCC, this value varied between 11.08 x 10^3 cells/ml and 1092.22 x 10^3 cells/ml with average number of 153.39 x 10^3cells/ml in cows without SM. In cows with SM, SCC varied between 445.83 x 10^3 cells/ml and 2868.25 x 10^3cells/ml, with average number of 724.83 x 10^3 cells/ml.

DISCUSSION

In this study, the cow level prevalence of SM and quarter level prevalence of SM were 51% and 27% respectively with *S. uberis* found in 35%, CoNS in 30% and *S. aureus* in 9%. In this research CoNS are identified on species level, and the most frequent isolate was *Staphilococcus haemolyticus* (10.34%), followed by *Staphylococcus simulans* and *Staphylococcus epidermidis* (both by 6.90%).

In the study of Sylejmani et al. (26) in Kosovo, the cow level and quarter level prevalence were lower (25,6% and 12.2% respectively) but the prevalence of contagious pathogens was higher. In that study *S. aureus* (28.6%) and *S. agalactiae* (14.3%) were the most isolated bacteria, and the environmental pathogens were identified to a lesser extent probably due to the targeted farms with smaller number of cows. Similar results were reported by Abdel-Rady and Sayed (7). In their research, they detected cow and quarter level prevalence of 19% and 5.7% respectively, with highest prevalence of *S. aureus* (52.5%) and *S. agalactiae* (16.25%). The difference in the results may occur due to the different sampling and interpretation of the results. Namely, in both studies the milk samples were from California mastitis test (CMT) positive quarters. According to Dohoo et al. (27) the cut-off for the CMT is 150.000 – 400.000 cells/ml and the weak positive sample is characterized by 300.000-1.000.000 cells/ml. This implicates that grading the CMT test as negative or weak positive is solely based on the experience of the assessor and can easily lead to misinterpretation. Tomazi et al. (28) found that the average number of the SCC in quarters infected by CoNS was 306.106 cells/ml, while Djabri et al. (29) demonstrated that the average SCC in quarters infected with *Corynebacterium bovis* and CoNS was 138.000 cells/ml and 105.000 cells/ml respectively. This implicates that a CMT negative quarter can also be infected, so by culturing milk only from a CMT positive quarter, the intra-mammary infection from CMT negative quarters (quarters infected with environmental pathogens or newly-infected quarters) can be easily overlooked.

The results of Pitkala et al. (22) demonstrate an overall quarter level prevalence of 33.5%, with CoNS, *Corynebacterium bovis* and *Staphylococcus aureus* isolated in 49.6%, 34.4% and 10% of quarters respectively. These authors used single sampling method but with lower bacteriological threshold of 5cfu/0,1ml and high SCC threshold of 300.000 cells/ml. However, they speculate that because of their SCC threshold that could be high and not sensitive enough, as well as the used single sampling method, some of the results could be misclassified.

The low sensitivity of a single sample method is also emphasized (5, 27). Persson et al. (5) concludes that the sensitivity of the detection of SM could be higher if cows are sampled repeatedly within a few days. This claim is in accordance with the guidelines of NMC for double or triple sampling in order to minimize the false positive and false negative assessment of the milk samples.

Regarding the quarter location and quarter level prevalence, this study found some differences which were not statistically significant. This is similar to the findings of Kocygit et al. (30) which determined higher prevalence of the rear quarters due to their lower set but with no statistical significance. However, these data are in concordance with the study of Slyzius et al. (31) where statistical significance among quarter location and quarter level prevalence was determined only when the difference in quarter milk production was above 11%.

As the primary aim of this study was the estimation of SM prevalence and the determination of the microbial panorama in SM infected quarters, the SCC was not considered as a crucial factor in the estimation of a positive and negative quarter. Nevertheless, the ranges and average values of SCC were determined in the bacteriologically positive and negative quarters. The SCC is the simplest, cheapest and most used method for estimation of SM in dairy cows (4, 7, 30) and international standard for estimation of milk quality/quarter health status (12). Generally, most of the authors indicate that quarters with SCC above 200.000 cells/ml can be considered as SM positive (4, 30, 32, 33). In our study, the SCC below 200.000 cells/ml was not always associated with culture negative quarter, and vice versa. According to Gianneechini et al. (1), the impossibility to isolate pathogens from quarters with SCC above 200.000 could be due to: spontaneous healing, the presence of few viable bacteria, used antibiotic therapy and loss of bacterial viability prior culture. According to our experience, this reason on our farms could be the lack of monthly records for SCC, absence of data for udder and/or quarter disease history, as well as prescribed and applied antimicrobial therapy. In addition, another reason could be that the microbiological analysis (in this study as well as general in our country) was conducted for the most common bacterial species affecting the udder, but not Mycoplasmas, viruses, Prototeca etc., as well as

the impossibility to isolate the pathogendue to other reasons (for example lower bacterial counts).

The quarters with SCC below 200.000 cells/ml and culture positive could be latently infected or infected with CoNS (26). Forsback et al. (3) demonstrated that there is a reversible pattern between isolated bacteria and SCC value during mastitis caused by *Staphylococcus aureus*, where low bacterial counts can be associated with high SCC and vice versa. This fact implies that low bacterial count below detectable numbers can lead to false negative results.

CONCLUSION

Although the results of this study represent a small scope of the herds and dairy cows in the Republic of North Macedonia, the high prevalence of SM in investigated farms could be some general indicator for the similar small farms. The displayed microbial diversity indicates lack of good farm management practices, housing hygiene, as well as health status and proper hygiene of the udder. Additionally, the high SCC in SM positive quarters could be a crucial factor in loss of milk quantity and quality. Therefore, further studies with bigger scope should be carried out in order to have better estimation of the SM prevalence in the country determining the significance of the SM in dairy sector on national level.

CONFLICT OF INTEREST STATEMENT

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article

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