Virulence Factors and Prevalence of Udder´s Pathogens in Dairy Cows during the Peripartal Period

Zuzana Farkašová[1*], František Zigo[2] and Zuzana Lacková[3]

[1] Department of Nutrition and Animal Husbandry, University of Veterinary Medicine and Pharmacy Košice, Slovakia
[*]*Corresponding author´s mail: zuzana.lackova [AT] uvlf.sk*

[2] Department of Nutrition and Animal Husbandry, University of Veterinary Medicine and Pharmacy Košice, Slovakia
Email: frantisek.zigo [AT] uvlf.sk

[3] Department of Nutrition and Animal Husbandry, University of Veterinary Medicine and Pharmacy Košice, Slovakia
Email: zuzana.farkasova [AT] uvlf.sk

ABSTRACT— ***Data presented in this study described the prevalence of udder pathogens and presence of virulence factors in staphylococci isolated from mastitis in dairy cows. The practical part of study was realized in five different dairy herds of Slovak spotted cattle breed located in Eastern Slovakia with conventional (non-organic) farming. At the beginning of lactation (during first two months after calving) were comprehensively investigated 960 cows from monitored herds. The comprehensive examination included clinical examination, sensory analysis of milk from fore stripping of each udder quarter, followed by assessment of the California mastitis test (CMT) and laboratory analyses of bacteria pathogens. Screening the health status of the mammary gland revealed that 314 cows (32.7%) had positive CMT score (1-3) for one or more quarters. Out of 230 infected milk samples, representing 24.0% of all dairy cows examined, were the most commonly isolated staphylococci (59.1% of positive findings), followed by E. coli (11.3%), streptococci Str. uberis (9.1%), Str. agalactiae (3.4%), and enterococci (6.1%). From 136 isolates of coagulase negative stafylococci (98 isolates) and S. aureus (38 isolates) were detected some virulence factors such as production of hemolysins (lysines ß and δ), gelatinase, biofilm, and hydrolyze of DNA. Isolated S. aureus, S. chromogenes and S. warneri had the most numerous representation of detected virulence factors, as demonstrated by the increased incidence of clinical forms of mastitis compared to less virulent strains.***

Keywords— Dairy Cows, Peripartal Period Mastitis, Biofilm, DNAse

1. INTRODUCTION

Bovine mastitis, the result of complex interactions among the host, environment and infectious agents. It is one of the most prevalent diseases of dairy cattle, and affects world dairy production, decreasing the quantity and quality of milk products. Mastitis differs from most other animal diseases in that several diverse kinds of bacteria are capable of infecting the udder. These pathogens invade the udder, multiply, and produce harmful substances that result in inflammation [3, 28].

To date, over 137 different organisms have been identified as being causative agents of bovine mastitis, including bacteria, viruses, mycoplasma, yeasts and algae but bacteria remain the principle causative agents (95% of all IMI) of such complex. Generally, every mastitis case is considered to be caused by one primary pathogen, because usually only one bacterial species is identified in milk samples from diseased glands. Nevertheless, simultaneous infections by two different pathogen species are not rare, and three pathogens may be found in a small proportion of cases [1, 26].

The bacteria causing the most common forms of mastitis may be considered within two groups. Contagious pathogens (e.g. *Staphylococcus aureus*, *Streptococcus agalactiae* or *Streptococcus dysgalactiale*). These organisms can survive and grow within the MG so that transmission of infection from infected to uninfected quarters and from cow to cow is most likely to occur during milking. Environmental pathogens thrive in the environment especially where cows' faeces are involved [6, 32].

In recent years, *S. aureus* and CoNS belong of the most common microorganisms causing mastitis in dairy cows. The manifestations of the inflammatory process caused by staphylococci are different, as they depend on the degree of reaction of the udder tissue to injury or infection. The clinical manifestations of mammary gland (MG) inflammation as well as its further course depend on the interplay between the innate resistance and adaptive immunity of the dairy cow and the concentration, and virulence of staphylococcal strains [16].

Generally, if the *S. aureus* is able to penetrate the teat in sufficient numbers the disease taken one of two clinical forms of IMI. Peracute staphylococcal mastitis can occur rarely, but especially in early lactation when the immune defenses of the cow are depressed. Although the cow with peracute *S. aureus* infection can be saved by an effective antibiotic, if caught in time, the quarter is almost invariably lost [4, 17].

The more common form of *S. aureus* infection is less severe but chronic. The affected cow may not appear ill and the affected quarter may not be painful. The foremilk may or may not show abnormalities. Treatment of *S. aureus* infection is complicated by the fact there are many strains and more and more of them are becoming resistant to more and more of the antibiotics within the veterinary armory. Their increased resistance is also due to the fact that, in addition to treating clinical cases of IMI, the common routine on farms is to dry dairy cows across the board with antibiotics. The studies confirm the increased resistance of both *S. aureus* and CoNS to those antibiotics, that are part of intramammary applicators used to treatment of dry dairy cows [10, 11].

Of this group, *E. coli* is the most important with multiple strains of varying pathogenicity for animals and humans. Others include *Streptococcus uberis*, coagulase-negative staphylococci (CoNS), *Corynebacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Proteus* spp., *Pasteurella* spp., *Listeria* spp., *Leptospira* spp., *Yersinia* spp., *Enterobacter* spp., *Brucella* spp. and *Mycobacterium* spp. [10, 27].

On the other side, CoNS are considered to be minor pathogens in dairy mastitis however, there is increasing work by authors to emphasize their role in the development of MG inflammation [8, 18, 29]. The increase of their occurrence in dairy farms occurs after the reduction of the occurrence of the main pathogens, the CoNS that are present are characterized by increased resistance to commonly used antibiotics and disinfectants. Compared to *S. aureus*, CoNS usually have a lower proportion of virulence factors but their essential factor of pathogenicity is the production of a biofilm and thus resist the applied disinfection and sanitation procedures. In addition, in other study, confirmed that the CoNS (*S. epidemidis, S. saprophyticus, S. hominis and S. aerletae* isolated from mastitic cows, were resistant to the antibiotics used and were able to produce some of the staphylococcal enterotoxins [18]. Other authors consider the ability to produce biofilm and lysine to be an important virulence factors that are responsible for the development of clinical forms of mastitis [8, 29].

The aim of this study was to monitor the occurrence of udder pathogens. Particularly in isolated staphylococci were determined the presence of selected virulence factors (formation of hemolysins, gelatinase, biofilm, hydrolyze DNA,) and their effect on the severity of mastitis in dairy cows.

2. MATERIAL AND METHODOLOGY

2.1. Monitored herds and udder health examination

The practical part of study was realized in five different dairy herds located in Eastern (4 herds) and Western (1 herd) Slovakia with conventional (non-organic) farming. Herds size ranged from 150 to 300 dairy cows of Slovak spotted breed between 1st - 4th lactation were used. From all monitored dairy farms, were investigated 270 cows from first, 215 cows from second, 175 cows from third, 153 cows from fourth, and 147 cows from fifth herd. Clinical examination of selected dairy cows, was carried out gradually with the collection of a mixed milk sample, after their transfer to the production group during the first two months of lactation.

A thorough evaluation of udder health included clinical examination, sensory analysis of milk from fore stripping of each udder quarter, followed by assessment of the California mastitis test (CMT) (Indirect Diagnostic Test, Krause, Denmark). Milk from every quarter was mixed with the reagent, and the result was scored as negative, trace, or positive (score 1–3) depending on the formation of gel in the milk sample according to Tančin [25].

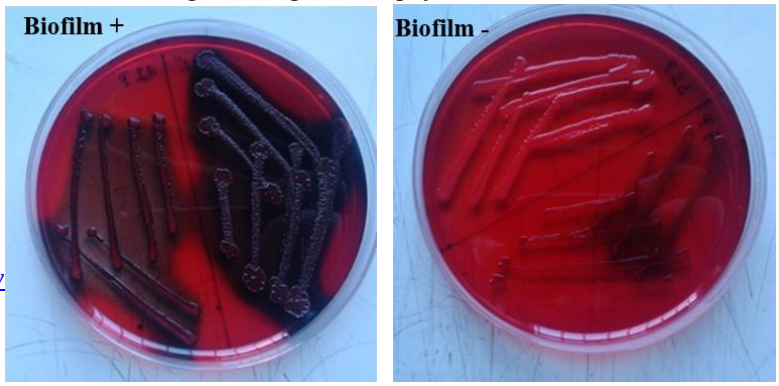
Subsequently, from the 960 examined cows were selected 314 cows with CMT score trace positive. From selected cows were aseptically collected 314 mixed milk samples for bacteriological cultivation in accordance with the guidelines of the [19]. The samples were cooled to 4 °C and immediately transported to the laboratory and analyzed on the following day.

2.2. Bacteriological culture

Milk inoculum (10 µl) of each sample was aerobically cultivated on the plates with 5% blood agar, at 37 °C for 24h.

The primocultivation and identification of *Staphylococcus* spp. was carried out according to the assessment growth of suspected bacteria on nutrient agars (5% of blood Agar, N° 110, Baird-Parker agar, Brilliance UTI Clarity Agar (OXOID

Figure 1. Biofilm production on Congo Red Agar for staphylococcal detection



Ltd., Basingstoke, Hants, UK). The pigment formation, catalase positivity, Gram positivity, creation of free or coupled coagulase, the ability of staphylococci to hydrolyze DNA, production of hemolysins, gelatinase and biofilm (Fig. 1) were determined according to studies [9, 20, 30]. Streptococci were determined by colony morphology and CAMP-reaction according to Holko [10]. Enterococci were confirmed by Gram-staining and growth of typical colonies on SlaBa-plates (Slanetz & Bartley, Medium, Oxoid Ltd., Basingstoke, England). A milk sample was classified as positive if more than three colonies from one type of colony-forming unit (CFU) of *S. aureus* or *Str. agalactiae* was isolated. For other bacteria, the presence of more than five CFUs was needed for positive classification. Samples were classified as contaminated if three or more bacterial types were isolated from one milk sample and growth of a major udder pathogen was not identified. The identification of each species was made by Staphy test, Strepto test, and resp. Entero test using the software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic) with a probability of correct designations of the kind above 90%.

3,44

2.3. Statistical analysis

Data were entered in to Microsoft Excel 2007® (Microsoft Corp., Redmond, USA) and analyzed using Excel, State 11, and SPSS version 20 (IBM Corp., Armonk, USA). The dependence of production of virulence factors in the four most frequently isolated bacterial pathogens among the type of mastitis in cows were statistically analysed using the Chi-square test [13]. The dependence of the individual signs was tested at a significance level $\alpha = 0.05$, with critical value = 7.815.

3. RESULTS AND DISCUSSION

An examination of five dairy cow herds showed, that from the 960 dairy cows examined during the first two months of lactation, 314 cows (32.7%) had CMT score (1-3) for one or more quarters. Of the mixed milk samples taken from selected dairy cows based on CMT scores, 230 were (73.2%) identified bacterial agents of mastitis causing a clinical or subclinical form of mastitis, and 84 (26.8%) samples were identified as negative or contaminated. The results of cultivation of microorganisms are shown in Table 1. Out of 230 infected milk samples (23.9%) of all dairy cows examined, and 73.2% of the samples taken were the most commonly isolated staphylococci. Mixed infection of two pathogens was identified in 16 cases with the combination of a major udder pathogen. The CoNS represented the most commonly detected bacteria (42.6% of positive findings); *S. aureus* (16.5%) were the second most abundant pathogens followed by *E. coli* (11.3%), streptococci (*Str. uberis*: 9.1%, *Str. agalactiae* (3.4%) and enterococci (6.1%).

Table 1: Pathogens isolated from milk samples of four monitored dairy herds

Pathogens	**Number of isolates**	**%** (n=230)	**Clinical IMI[1] %** (n=230)	**Subclinical IMI[1] %** (n=230)
CoNS	98	42.6	17.4	25.2
S. aureus	38	16.5	10.0	6.5
Escherichia coli	26	11.2	3.0	8.1
Streptococcus uberis	21	9.1	6.1	3.0
Streptococcus agalactiae	8	3.4	3.0	0.4
Streptococcus spp.	10	4.3	1.7	2.6
Enterococcus spp.	14	6.1	2.2	3.9
Mixed infection	16	6.9	1.3	5.6
Total	**230**	**100**	**44.7**	**55.5**

IMI[1] – intramammary infection

Our results are consistent with the similar study, which recorded a high incidence of CoNS and *S. aureus* isolated from milk samples during the examination of 42 dairy farms in the west of Slovakia. The CoNS represented 35.9% of positive findings and were the most commonly detected bacteria [10].

Among the most serious causative agents of mastitis was *S. aureus*, which was isolated in 23 clinical and 15 subclinical cases of mastitis. Eight species were isolated from CoNS, with the following recorded as the most numerable species: *S. chromogenes* (22.4%), *S. warneri* (20.4%), *S. xylosus* (18.4%), *S. epidermidis* (9.1%) and *S. haemolyticus* (7.1%). The representation of CoNS on the individual forms of IMI was different. Most frequently were detected cases of subclinical mastitis (58), caused predominantly by *S. warneri*, *S. xylosus* and *S. epidermidis*. Clinical mastitis were detected in 40 cases, caused by *S. chromogenes*, *S. haemolyticus*, *S. warneri*, and *S. xylosus.*

In compare to our results the researchers reported, that the *S. haemolyticus*, *S. chromogenes*, *S. warneri* and *S. xylosus* were isolated as dominant strains of CoNS isolated from mastitis in cows. CoNS was mainly due to clinical mastitis associated with significantly reduced milk production, an increase in SCC [23, 24].

For the individual virulence factors (Table 2), co-production of lysines ß and δ observed in 7 species, single lysine δ in nine species of staphylococci. Production of DNAse was detected in *S. aureus, S. chromogenes, S. warneri, S. xylosus,* and *S. haemolyticus* as well as production of gelatinase, except for *S. xylosus* and *S. haemolyticus*. The stafylococci *S. aureus, S. chromogenes* and *S. warneri* had most numerous representation of virulence factors (production of lysines, gelatinase and biofilm, the ability to hydrolyze DNA) what are resulted as increasing incidence of clinical cases of mastitis in compare to less virulent strains. From all 40 clinical IMI, in 35 cases the production of lysines, in 23 hydrolysis of DNA, 17 cases with production of gelatinase, and 33 cases with biofilm production were detected. Totally, the production of biofilm we found in 50 isolates, whereas in *S. capitis* the production of biofilm has not been detected. On the significance level of $\alpha = 0.05$ was confirmed the independence the production of virulence factors on type of mastitis in cows, in four strains of staphylococci *(S. aureus, S. chromogenes, S. warneri, S. xylosus).*

Table 2: The role of CoNS in severity of mastitis and the occurrence of selected virulence factors

***Staphylococcus spp.*/number**	IMI[1]/number	hemolysins[2]	**DNAse[3]**	**gelatinase**	**biofilm**	**Test* G**
S. aureus (38)	clinical (23)	9α+β/7α/4δ	14	11	12	1.089*
	subclinical (15)	3α+β/4α	8	7	5	
S. chromogenes (22)	clinical (13)	4β+δ/4δ	3	4	7	0.332*
	subclinical (9)	2δ	1	1	3	
S. warneri (20)	clinical (9)	2β+δ/4δ	2	2	5	1.578*
	subclinical (11)	3β+1δ	2	0	2	
S. xylosus (18)	clinical (8)	2β+1δ/3δ	2	0	5	1.601*
	subclinical (10)	2β+1δ	0	0	4	

IMI[1] – intramammary infection/number of isolates and their incidence on severity mastitis; lysines[2] - production of hemolysins type α,β or δ; DNAse[3] - ability of staphylococci to hydrolyze DNA; *Chi-quadrate test (on significant level $\alpha = 0.05$ (5%) critical value $\chi^2 = 7.815$), * – independence of individual sign on significant level $\alpha = 0.05$ was not rejected.

Biofilm production by the staphylococcal strains is consider an important virulence factor responsible for adhesion of these microorganisms with living or non-living surfaces [22].

The bacteria carrying this typical peculiarity are highly resilient to antibiotics. The intramammary infection due to biofilm producers *S. aureus* or CoNS is difficult to treat even with intramammary antibiotics so proper considerations should be given to the infections produced by biofilm producing bacteria [5, 12, 14]. Generally, staphylococcal strains, which produce biofilm, lead to chronic mastitis after unsuccessful treatment, especially with beta-lactam antibiotics in dairy animals [2, 7, 31].

Other author in his study of staphylococci isolated from mastitis milk in cows reported that the biofilm production and resistance to antibiotics were most frequently virulence factors in strains isolated from clinical IMI. Increasing biofilm production was evident in strains from repeat and chronic cases of mastitis [15]. Similar study report, that the production of biofilm was determining factor of pathogenity of *S. epidermidis* in hospital infections, what has been confirmed in 3 isolates in our study [21].

3. CONCLUSION

The study showed that most frequently isolated udder pathogens were CoNS, followed *S. aureus*, *E. coli* and streptococci. Coagulase negative staphylococci such as *S. chromogenes, S. warneri* and *S. xylosus* isolated from clinical mastitis indicated highest degree of pathogenity in production of more virulence factors (lysines, DNAse, gelatinase, biofilm). On the significance level of $\alpha = 0.05$ the dependence the production of virulence factors in the four most frequently isolated staphylococci on type of mastitis in cows was rejected. From the results manifests that the impact of CoNS is increasing on the occurrence of IMI, probably because prevalence of others major pathogens (*Str. agalactiae* or *Str. dysgalactiae)* is decreasing. In order to reduce occurrence and resistance of bacterial agents causing the predominantly chronic IMI, it is necessary to base the obtained antibiogram results on monitored farms as well as selective therapy of dairy cows during dry period.

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