

MASTITIS PATHOGENS AND THEIR RESISTANCE AGAINST ANTIMICROBIAL AGENTS IN HERDS OF DAIRY COWS SITUATED IN MARGINAL PARTS OF SLOVAKIA

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ABSTRACT

Marginal regions are relatively large part of the area Slovakia which in terms of the economy breeding ruminants can efficiently produce animal commodities only occasionally. Geographic, social and economic stability of these regions is strongly influenced by breeding of ruminants with market milk production. Mastitis is a disease complex that assumes highest clinical and economic significance in milk animals particularly medium to high yielding dairy cattle, usually in and around periparturient period. The objective of this study was to evaluate the effectiveness of different antibiotics against mastitis causing microorganisms during first month of lactation in two herds of 230 and 310 dairy cows situated in marginal parts of Slovakia. Milk samples from quarters were cultured and identified bacteria were subjected to antimicrobial susceptibility test by disc diffusion method to a large number of antibiotics. The prevalence of mastitis in the monitored herds of dairy cows was 26.1% to 17.6%, respectively. A total of 1663 milk samples from udder quarters were investigated, 446 (21.3%) samples were positive. No pathogens were isolated from 1663 (78.4%) milk samples. From all tested bacteria *Staphylococcus* spp. and *Streptococcus* spp. which were isolated from subclinical and clinical mastitis, were found amoxicillin + clavulanat and tetracycline to be most effective drug followed by ceftiofur and rifaximin. The significant difference was confirmed between the *Staph. aureus* and coagulase-negative staphylococci (CoNS) isolates with respect to their susceptibility to the various antibiotics. Antibiotic susceptibility tests should be done to determine the effectiveness of drug that can be used for successful treatment of diseases. Proper isolation and identification of the causative organism play significant role in prevention and control of the diseases.

Keywords: dairy cows; mastitis; resistance; *Staphylococcus aureus*; *Streptococcus agalactiae*

INTRODUCTION

Economic, social and geographic stability of marginal regions is strongly influenced by the existence of agriculture and especially livestock production (cows and sheep represent 75% of animal production of these areas). Products from dairy ruminants are unique, especially in the field of rational nutrition of consumers. Many of the milk products and specialties can be included among the functional foods (Vršková et al. 2015).

Inflammation of the mammary gland - mastitis is the most significant disease of dairy herds, has huge effects on farm economics due to reduction in milk production and treatment costs (Østerås 2006; Pyörälä and Taponen, 2009).

In Slovakia, costs of clinical mastitis is estimated about 150 – 200 € per cow/year, above the desired baseline in European dairy herds (Tančin et al. 2006).

The estimated loss of milk per cow per one lactation cycle is 70% of the total losses and the cost of cows lost due to premature culling is 14%, while the cost of milk downgraded/ discarded due to mastitis is 7% and the cost

incurred on medical treatment and other veterinary expenses amount to 8%, of the total losses, reported worldwide. Mastitis low prevalence herds can save up to 25% cost on losses than the high prevalence herds (Kader et al. 2002; Shaheen et al. 2016).

Bovine mastitis, is predisposed by several epidemiological risk factors that play significant role in causing mammary incompetence to protect it from the invasion of infectious agents. These should receive due consideration in the course of developing an integrated mastitis control programme. The risk factors include the host factors, environmental factors and the pathogen factors (Vasil' et al. 2004; Pitkälä et al. 2001).

Mastitis, can be caused by a wide range of organisms, including gram-negative and gram-positive bacteria, mycoplasmas and algae (Malinowsky, 2000; Malinowsky and Gajewski, 2009; Zadoks et al. 2011; Pitkälä et al. 2004).

Many microbial species that are common causes of bovine mastitis, such as *Escherichia coli*, *Streptococcus*

agalactiae, *Staphylococcus aureus* and *Klebsiella pneumoniae*, also occur as commensals or pathogens of humans whereas other causative species, such as *Streptococcus uberis*, *Streptococcus dysgalactiae subsp. dysgalactiae* or *Staphylococcus chromogenes*, are almost exclusively found in animals (Zadoks et al. 2011; Kmet' et Bujňáková, 2018).

Antimicrobials are routinely used for treatment of dairy cattle affected with clinical and subclinical infections (Aarestrup, 2005; Medved'ová et al. 2009).

The use of antimicrobials has, over time, increased the number of antimicrobial-resistant microbes globally, and any use of these agents will to some extent benefit the development of resistant strains and also inappropriate usage of antimicrobials such as wrong dose, drug or duration may contribute the most to the increase in antimicrobial resistance without improving the outcome of treatment (Williams, 2000; Idriss et al. 2014).

Scientific hypothesis

The aim of this study was to evaluate the effectiveness of different antibiotics against mastitis causing microorganisms during first phase of lactation in two dairy herds cows situated in marginal parts of Slovakia.

MATERIAL AND METHODOLOGY

Animals and milking

The study was conducted according to good veterinary practice. The practical part of study was realized in two different farms situated in marginal parts of Slovakia (Orava, Zemplín) with standard zootechnic and zoohygienic conditions. Herd size ranged from 230 (A) to 320 (B) dairy cows of Holstein breed between 2nd - 4th lactation was used, respectively. Dairy cows from both farms were kept in a free housing system with a separate calving barn and equipped with individual boxes with bedding and were allowed *ad libitum* access to water. All herds were fed total mixed ration based on grass silage, maize silage and concentrate.

All cows were milked twice daily. The cows from herd A were milked in tandem parlor DeLaval 2x5 (Tumba, Sweden). In parallel parlor Boumatic 2x12 Xpressway (Wisconsin, USA) were milked cows from herd B. The average milk yield ranged from 7300 (herd A) to 7800 l (herd B) per year. Blanket dry cow therapy was implemented in all herds.



Figure 1 Quarter milk samples collection, bacteriology analysis and antimicrobial susceptibility test by disc diffusion method.

Samples collection

A complex examination of the health status of the animals included clinical examination of the mammary gland, cytological examination the first portion of milk, NK-test reaction with subsequent collecting of milk samples (quarter samples) for bacteriological examination, and subsequent cultivation and identification of pathogenic bacteria (Figure 1). Quarter milk samples were collected aseptically from 2120 quarters (530 cows) a first month after calving. Eleven quarters were atrophic. Before sampling, the first streams of milk were discarded, and teat ends were disinfected. The 10 ml of the milk was collected into sterile tubes. The samples were cooled and immediately transported to the laboratory.

Laboratory analyses

Bacteriological examinations were performed according to commonly accepted rules Malinowski and Klossowska (2002). Milk samples (0.05 mL) were inoculated onto blood agar (Oxoid, UK) and cultivated at 37 °C for 24 h. Based on the colony morphology and by Gram staining, bacteria *Staphylococcus* spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspect colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriaceae* spp. were isolated on blood agar, cultivated at 37 °C for 24 h and detailed identified biochemically using the STAPHY-test, STREPTO-test, resp. ENTERO-test and identification by software TNW Pro 7.0 (Erba-Lachema, CZ).

Health udder and individual forms of mastitis (subclinical, subacute and acute) based on clinical signs, NK-test scores and bacteriological examination of milk samples were classified according to Jackson and Cockcroft (2002).

Antimicrobial susceptibility test

The bacteria *Staphylococcus* spp. and *Streptococcus* spp. isolated through microbiological procedures were subjected to antimicrobial susceptibility test by disc diffusion method to identify the most effective drugs for mastitis treatment in the study area (Hameed et al. 2008).

The sensitivity against penicillin, amoxicillin, amoxicillin + clavulanat acid, ceftiofur, cloxacillin, enrofloxacin, lincomycin, neomycin, nafpenzal, rifaximin, streptomycin and tetradelta were determined on Mueller Hinton agar as described by National Committee for Clinical Laboratory

Standards (NCCLS, 2002). The results were obtained by measuring the diameter of the growth inhibition zone around the antibiotic disc for each isolated bacterial strain and recorded as sensitive, intermediate and resistant.

Statistical analysis

Statistical analyses were performed using Graph-Pad PRISM 6.0 (GraphPad Software Inc., USA). Differences in incidence of mastitis among herds and in the distribution of the antibiotic resistant bacteria were statistically analyzed using Chi-square test. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The prevalence of mastitis in the monitored herds of dairy cows was 26.1% to 17.6%, respectively (Table 1).

The economic losses are more associated with subclinical mastitis which is 40 % more prevalent than clinical mastitis (Hortet et al. 1999).

However, the cost of treatment of subclinical mastitis is much low compared to that of clinical mastitis accounting for 10 – 20 times higher (Shaheen et al. 2016).

Occurrence of subclinical forms in our study were from 10.4% to 7.8%, respectively. Subclinical mastitis is difficult to detect due to the absence of any visible indications, and it has major cost implications. Chronic mastitis is a rarer form of the disease but results in persistent inflammation of the mammary gland. Currently, milk quality payments are based on somatic cell counts (SCC), and elevated levels result in reduced payments. This, in addition to reduction in milk volume and treatment costs, significantly affects farm incomes (Allore et al. 1998; Seegers et al. 2003).

The differences in incidence of subacute forms mastitis in examined quarters among herds were partially statistically significant ($p < 0.05$). The incidence of subacute mastitis varied among herds from 9.0 % to 4.1 %, respectively. Occurrence of clinical mastitis were from 4.0 % to 1.9 %, respectively (Figure 2).

The nutrient composition of milk is ideal medium for bacterial growth and therefore it can be considered one of the most perishable agricultural products because it can so easily be contaminated. Raw cow and sheep milk may contain microorganisms which can cause food borne disease (Zajác et al. 2012).

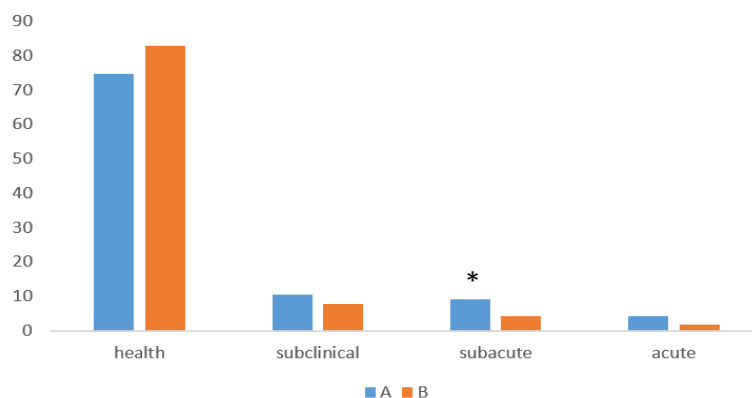


Figure 2 Comparison of individual forms of mastitis. Note: A – dairy herd with prevalence of mastitis 26.1%, B – dairy herd with prevalence of mastitis 17.6%.

Table 1 Incidence of mastitis in examined herds.

Monitored herds	A		B		Total
	n	%	n	%	n
Healthy quarters	668	73.9	995	81.8	1663
Positive quarters	232	26.1 ^a	214	17.6 ^b	446
Infected quarters	207	22.8 ^a	182	14.9 ^b	389
Reject quarters	4	0.4	7	0.6	11
All examined quarters	904	100	1216	100	2120
Total dairy cows in herd	226		304		530

Note: a, b – values in row with different superscript letters differ significantly at $p < 0.05$.

Table 2 Isolated microorganisms from infected quarters in monitored herds.

Isolated microorganisms	n	subclinical		subacute		acute	
		A %	B %	A %	B %	A %	B %
<i>Staph. aureus</i>	49	14.3	6.1	20.4	10.2	36.7	12.2
<i>Str. uberis</i>	17	11.8	-	41.2	-	47.5	-
<i>Str. agalactiae</i>	31	9.7	6.4	35.5	12.9	22.6	12.9
<i>Streptococcus spp.*</i>	25	24.0	12.0	36.0	12.0	-	16.0
CNS*	131	16.7	34.4	12.2	9.9	5.3	3.1
CPS*	14	28.6	21.4	35.7	14.3	-	-
<i>E. coli</i>	36	38.9	25.0	27.8	8.3	-	-
<i>Enterococcus spp.</i>	30	16.7	50.0	16.7	20.0	-	-
<i>Bacillus spp.</i>	28	46.4	17.9	17.9	7.1	-	10.7
<i>Enterobacter aerogenes</i>	17	52.9	17.4	17.4	-	-	11.7
<i>Others*</i>	11	54.5	27.3	18.2	-	-	-
Total	389	24.1	18.3	21.3	9.8	10.3	5.9

Note: n – number of isolated bacteria, Others* – *Proteus spp.*, *Aerococcus spp.*, CPS* – *S. hyicus*, *Str. spp.** - *Streptococcus faecalis*, *Str. dysgalactiae*, *Str. suis*, CNS* – *S. haemolyticus*, *S. chromogenes*, *S. xylosum*, *S. epidermidis* and *S. warneri*.

A total of 1663 milk samples from udder quarters were investigated, 446 (21.3 %) samples were positive. No pathogens were isolated from 1663 (78.4 %) milk samples.

Several authors in their studies from Finland and Norway recorded, that the species of *Staphylococcus spp.* belongs to general aetiological agents of intramammary infections in ruminants (*S. aureus* in clinical and CoNS in subclinical cases) (Pittkala et al. 2004; Pyörälä and Taponen, 2009).

From the CoNS are more frequently *S. xylosum*, *S. epidermidis* and *S. chromogenes* (Østerås et al. 2006), what are also determined in our study.

It is well known that (CoNS) are the most important bacteria involved in subclinical bovine mastitis (Pyörälä and Taponen, 2009), alongside *Staphylococcus aureus* (Malinowski and Gajewski, 2009).

Their resistance to antimicrobial agents is common due to the high antibiotic pressure in conventional dairy farming. Usually different CoNS species from bovine milk differ significantly in their phenotypic and genotypic antimicrobial resistance profile, which is important for udder health management (Sampimon et al. 2011).

Enterococci, on the other hand, have only limited clinical importance in dairy farming, but their ubiquitous nature

and frequent carriage of resistance genes is a reason for concern (Zadoks et al. 2011).

In addition to staphylococcus, the main mastitis pathogens are *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. In our study, many infections caused by *Streptococcus uberis* and *Streptococcus agalactiae* were subacute and acute (Lalrintluanga et al. 2003).

In recent years, reported *Streptococcus uberis* incidence has increased with 16 cases/100 cows/year reported for 2005 (Bradley et al. 2007).

Bovine mastitis is the single most common cause for antibacterial use in lactating dairy cattle. Treatment of this disease is also the most common cause of illegal antibacterial residues in marketed milk (Muhamed et al. 2012).

Antibacterial therapy of bacterial induced diseases in cattle has been incriminated as a catalyst for resistance in bacteria isolated from treated animals, other animals within the herd, and food derived from cattle for human consumption (Foltys and Kirchrneová, 2005; Bengtsson et al. 2009).

The resistance in tested isolates are described in Table 3. From all tested bacteria *Staphylococcus spp.* and *Streptococcus spp.* were found Amoxicillin + clavulanat

Table 3 Frequency of susceptibility of *Staphylococcus* spp. and *Streptococcus* spp. to antibiotics.

Bacterial strains	<i>Staphylococcus aureus</i> (49)			CNS (131)			<i>Streptococcus agalactiae</i> (31)			<i>Streptococcus uberis</i> (17)		
	S	IS	R	S	IS	R	S	IS	R	S	IS	R
Penicillin	37	4	8	112	6	13	27	2	2	15	2	0
Ampicillin	41	2	6	116	4	11	27	3	1	10	1	1
Amoxicillin	40	3	6	121	2	8	26	3	2	13	2	2
Amox. + clav.	48	0	1	126	2	3	31	0	0	16	1	0
Ceftiofur	46	0	3	123	2	6	29	2	0	17	0	0
Cloxacillin	38	4	7	121	4	6	29	0	2	14	1	2
Enrofloxacin	39	2	8	117	7	7	27	2	2	14	2	1
Lincomycin	41	3	6	118	5	8	26	2	3	13	2	2
Neomycin	38	4	7	119	4	8	25	3	3	11	3	3
Nafpenzal	42	2	5	122	2	4	30	0	1	16	1	0
Rifaximin	44	2	2	123	3	3	28	1	2	16	1	0
Streptomycin	36	3	11	116	8	7	21	4	6	10	3	4
Tetradelta	47	0	2	128	1	3	31	3	0	16	0	1

Note: R – resistant, IS – intermediate sensitive, S – sensitive.

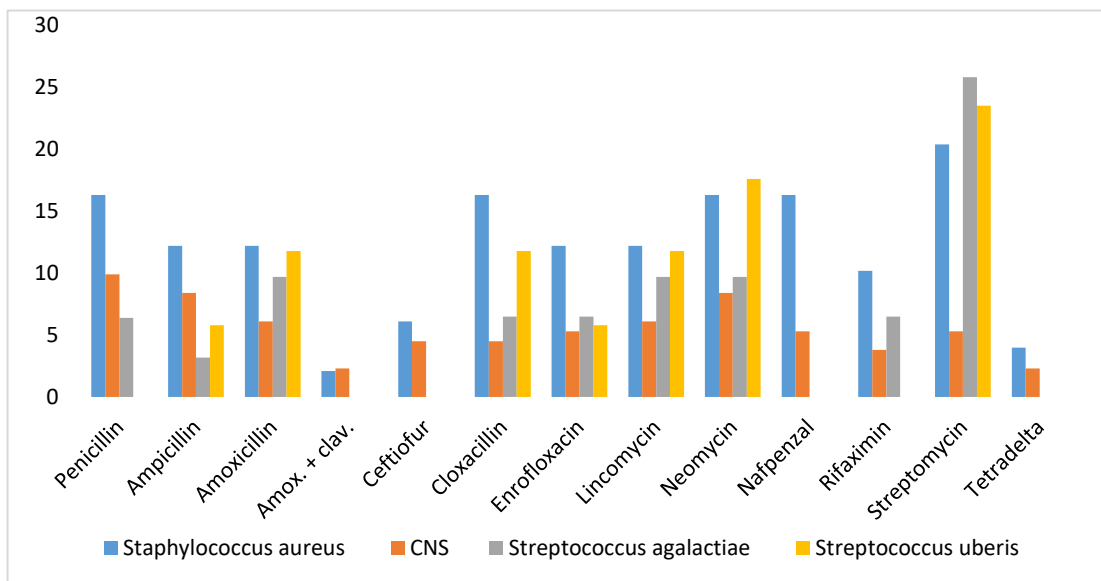


Figure 3 Difference between the pathogenic bacteria with respect to their susceptibility to the various antibiotics (%).

and tetradelta to be most effective drug followed by ceftiofur and rifaximin. On the contrary, antibiotics showing higher rate of resistance patterns were streptomycin, amoxicillin and penicillin. The significant difference ($p < 0.05$) was confirmed between the *Staph. aureus* and CoNS isolates with respect to their susceptibility to the various antibiotics. There is no significant difference in the tested *Str. uberis* and *Str. agalactiae* (Figure 3).

Our results are consistent with the work Kirkan et al. (2005), where 300 cases of mastitis were isolated 60 bacteria of CoNS (20.0%), which showed resistance to penicillin and streptomycin. Staphylococci were mostly

susceptible to antimicrobials tested but, Muhamed et al. (2012) found that *Staph. aureus* was resistant to penicillin and streptomycin (41.4% and 25.6% respectively).

Similar results were obtained by Sumathi et al. (2008) where *Staphylococcus* and *Streptococcus* spp. were resistant to streptomycin and penicillin. Those results are in accordance with our findings.

Vasil' (2009) tested isolated strains of *Streptococcus* spp. and CoNS and has found that *Strep. agalactiae* strains were sensitive to antibiotics except to penicillin, ceftiofur, while *Strep. uberis* was a complete sensitive to a combination of amoxicillin + clavulanat and ampicillin, followed by cefalotin, lincomycin, whilst it is resistant to

streptomycin, novobiocin and neomycin. Tested strains of CoNS were sensitive to a combination of amoxicillin + clavulanat and resistant to streptomycin and penicillin.

These results are in accordance with our findings that CoNS, *Strep. agalactiae*, *Strep. uberis* were sensitive to amoxicillin + clavulanat and tetracycline.

CONCLUSION

The results of our study showed that the incidence of subclinical and subacute mastitis at the start of lactation in monitored dairy herds is high. CoNS were the most frequently isolated from subclinical mastitis cases; however, clinical mastitis caused by the contagious pathogens *Staph. aureus*, *Str. agalactiae* and *Str. uberis* are still a problem and play an important role in dairy herds in marginal parts of Slovakia.

Antibiotic susceptibility tests should be done to determine the effectiveness of drug that can be used for successful treatment of diseases. In our study combinations of amoxicillin plus clavulanat acid, tetracycline and ceftiofur were the most effective antibiotics for control of bovine mastitis.

Antimicrobial resistance surveys in dairy production are mostly focused on udder pathogens and milk samples from drug treated animals. However, it is also important to evaluate the presence of resistant bacteria in regularly collected raw milk samples from clinically healthy animals, in order to assess the potential spread of resistant strains from raw material to dairy products.

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