Microbiological Studies on the Prevalence of *Staphylococcus* Spp., Involved in the Etiology of Mastitis in Cattle and their Susceptibility to Antimicrobial Agents

Mariana Nikolova[1], Valentina Urumova[1]

[1]Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Keywords: *mastitis, cattle, staphylococci, resistance.*

Abstract. *For the period between June 2020 and -March 2022, a total of 8 dairy cattle farms were surveyed in terms of the prevalence of clinical and subclinical mastitis. Four of them were located in Northern Bulgaria (Targovishte, Shumen and Dobrich districts) and another four in Southern Bulgaria (Stara Zagora, Plovdiv and Haskovo districts). In these target farms, a rapid mastitis screening test was initially performed to detect the presence of subclinical mastitis or samples with high somatic cell counts. A total of 312 milk samples were obtained from milk quarters who reacted with 3+ or 4+ to which 34 samples of inflammatory exudate from cows with clinical mastitis were added.*

During the microbiological investigation of the 346 samples, 272 of them (79.1%) were bacteriologically positive. Streptococcus spp., were isolated from 151 samples (55.5%). The second most common species was Staphylococcus spp., detected in 110 (40.4%) of the tested samples. In total, the Gram-positive cocci finding exceeded 95% of the microbial species. The remaining 11 (4.0%) isolates belonged to another 6 taxa. These included four strains identified by prior phenotypic identification as Trueperella pyogenes, three Escherichia coli isolates, and one strain of each Pasteurella multocida, Nocardia asteroides, Klebsiella pneumoniae, and Acinetobacter spp.

Staphylococci were also studied for their sensitivity to 11 chemotherapeutic agents. The highest percentage of resistance (50%) was determined to lincomycin, followed by that to tetracycline (37.3%), and beta-lactams ampicillin (24.5%), and oxacillin (13.6%). The resistance rates to cefoxitin and cephalotin were 5.5% and 0.9% respectively. Also, lower values of resistant strains were observed for the combination of trimethoprim/sulfonamides (7.3%), ciprofloxacin (1.8%), and rifampicin (0.9%). Resistance to gentamicin and amoxicillin/ clavulanic acid was not established. Minimum inhibitory concentrations were determined for the studied chemotherapeutics, with the highest MIC_{90} *values of 128 μg/mL and 2 μg/mL for tetracycline and lincomycin, and the lowest* MIC_{90} *values of 0.001 μg/mL for rifampicin and ciprofloxacin, respectively. A* MIC_{90} *of 0.125 μg/mL was obtained for oxacillin, 1.5 μg/mL for trimethoprim/sulfonamides and 1.0 μg/mL for ampicillin, cephalotin and cefoxitin.*

Introduction

Staphylococci are among the commonest agents of bovine mastitis, causing substantial economic losses and threatening public health. Apart the economic status of an affected farm, mastitis also influences the quality of milk, and respectively the health of offspring. On the other hand, the spread of multi-resistant microbial strains involved in mastitis etiology is also an important health issue related to animal welfare and risks for human health through the food chain.

Subclinical mastitis is one of the most frequent form of bovine mastitis, prevalent in many geographic regions, increasing milk somatic cell counts and altering milk physical and chemical properties (Abebe *et al.*, 2016; Jagielski *et al.*, 2014; Léon-Galvan *et al.*, 2015; Salvador et al., 2014).

The spread of resistance to chemotherapeutics used for treatment of mastitis is a serious challenge for control of these infections, and also a specific process influencing the therapeutic approach to affected populations (Sakwinska *et al.*, 2011; Silveira- Filho *et al.*, 2014; Vakkamäki *et al.*, 2017). The monitoring of antimicrobial resistance is beneficial for objective decision-making on the therapy and prevention of mastitis, and at the same time demonstrates the possible trends of resistance development, which is important for the correct use of antimicrobial drugs in veterinary practice (Mader *et al.*, 2021). The monitoring of resistance to beta-lactam chemotherapeutics is of particular interest, as they are frequently used for treatment of bovine mastitis, and some pathogenic strains, e.g. methicillin-resistant staphylococci have a public health impact as well (Schnitt and Tenhagen, 2020). The production of beta-lactamases is the commonest mechanism of resistance against beta-lactams among both Gram-negative and Gram-positive bacteria (Livermore and Brown, 2001).

Corresponding to Valentina Urumova, Trakia University Faculty of Veterinary Medicine Department of Veterinary Microbiology, Infectious and Parasitic Diseases Stara Zagora, Bulgaria.
E-mail: valentina_62@abv.bg

The aim of the present investigation was to perform a two-year survey on the prevalence of the most prevalent etiological agents of bovine mastitis and to analyze their sensitivity to most commonly used chemotherapeutic drugs.

Material and methods

From June 2020 to March 2022, a total of 8 dairy cattle farms were surveyed for prevalence of clinical and subclinical mastitis. Of them, four were located in North Bulgaria (2 in Targovishte district, 1 in Shumen and 1 in Dobrich districts); the other four were in South Bulgaria (Stara Zagora, Plovdiv and Haskovo districts). Initially, the cows at the farms were screened with the rapid Kerba mastitis test to detect subclinical mastitis or samples with high somatic cell counts. A total of 312 milk samples were collected from milk quarters having reacted with 3+ or 4+. To them, 34 samples of inflammatory exudate from cows with clinical mastitis were added.

All milk samples were cultured aerobically on Columbia blood agar (5% sheep red blood cells) and McConkey agar at 37°C for 24– 48 hours. The samples were determined as contaminated after the detection of growth of more than two morphotypes and absence of the specific growth of the main etiological agents. Strains from the positive samples were the first type depending on the expression of haemolytic activity: α-, β-, or double haemolytic zone, Gram staining, production of catalase and oxidase, free coagulase production and mannitol utilisation.

DNA extraction from suspect colonies of both coagulase-negative and coagulase-positive staphylococci was done with DNeasy Blood Tissue kit (Qiagen, Germany) and the identification of staphylococci was performed with commercial Microbial DNA qPCR assay kits- Qiagen, Germany. The emphasis in this study was on the genetic determination of *Staphylococcus epidermidis* and *Staphylococcus aureus*. The genetic assays employed TaqMan probes for detection of amplification regions determining the species affiliation of staphylococci, labelled with FAM reporter dye and ROX Reference Dye (passive reference dye). Amplification reactions were run in STRATAGENE Mx3000P qPCR (Agilent Technologies, USA). The reaction protocol was as followed:

Table 1. PCR protocol

Components	n=1
PCR water	6.5 µL
Master mix	12.5 µL
qPCR assay	1 µL
Mastermix volume	20 µL
DNA tamplate	5 µL
Total volume	25 µL

The temperature region of amplification reaction comprised: the initial step of denaturation at 95°C for 10 minutes, two steps of 40 cycles each consisting of denaturation (95°C for 15 sec), and annealing/elongation (60°C for 2 min).

For phenotype analysis of resistance of isolated staphylococci to antimicrobial drugs, the disk diffusion method and the method for determination of minimum inhibitory concentrations (MIC) (E-test, Hi Comb™) were used. The disks and test strips were produced by Himedia Biosciences, India. MICs of lincomycin were determined by the dilution in Muller-Hinton agar method. Lincomycin was provided by the Veterinary Preparations Enterprise in Zavet, Razgrad district, Bulgaria. The sensitivity of staphylococci was tested to eleven chemotherapeutical drugs from several classes. The used antibiotic disks included beta-lactams: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), oxacillin (1 µg), cephalothin (30 µg) and cefoxitin (30 µg); aminoglycosides-aminocyclitols: gentamicin (10 µg), tetracyclines: tetracycline (30 µg), fluoroquinolones: ciprofloxacin (5 µg), as well as lincomycin (15 µg), rifampicin (5 µg) and the combination trimethoprim/sulfonamide (23.75/1.25 µg). The reference strain *Staphylococcus aureus* ATTC 25923 was used as control in the phenotype analysis of resistance. The strains were interpreted as resistant or sensitive based on critical thresholds recommended by EUCAST (2022).

Statistical analysis of the data was performed with GraphPad Instat 3 software.

Results

The data from performed surveys are presented in Table 2. Of all 6568 tested milk quarters of dairy cows, 312 (4.7%) were found positive or suspect by the rapid mastitis test. The samples from all positive quarters were sent for microbiological examination together with another 34 samples from clinical mastitis.

The highest percentage of subclinical mastitis was found out in the farm in Mengishevo (29.8%), followed by the Karapelit farm (24.0%), the least prevalence was identified at the Kamburovo farm (7.4%), where the *Staphylococcus aureus* isolates were more spread (11 of the total 16 strains from bovine subclinical mastitis). The cows at the Karapelit farm exhibited a higher prevalence of coagulase-negative staphylococci (29 of 61 strains from bovine subclinical mastitis) followed by the Popovitsa farm (18 out of 28 strains from bovine subclinical mastitis). Staphylococci were the least prevalent at the Harmanli farm (2 of the total 4 strains from bovine subclinical mastitis).

A positive microbial finding was detected in 272 of all the tested 346 samples (79.1%). *Streptococcus* spp. was presented in 151 samples (55.5%). Second came the members of genus *Staphylococcus*. They were found out in 110 of the tested samples (40.5%). Of them, 78 (71.0%) were determined as coagulase-negative and 32 (29.1%) as coagulase-positive. In general, Gram-positive cocci exceeded 95% of all identified microbial species. Eleven isolates (4%) belonged to other 6 taxa. They included 4 strains phenotypically determined as *Trueperella pyogenes*, 3 *E.coli* strains, and a single strain of species *Pasteurella multocida*, *Nocardia asteroides*, *Klebsiella pneumoniae* and *Acinetobacter* spp.

Staphylococci were tested for their sensitivity to 11 chemotherapeutic drugs. Table 4 presents the results about the spread of antimicrobial resistance among staphylococci. The highest resistance rate (50%) was shown against lincomycin, followed by that against tetracycline (37.3%) and beta-lactams ampicillin (24.5%) and oxacillin (13.6%). The occurrence of resistance to cefoxitin and cephalothin was lower, 5.45% and 0.95%. respectively. Also, a lower prevalence was detected with respect to strains resistant to trimethoprim/sulfonamides: 7.3%, ciprofloxacin: 1.8% and rifampicin: 0.9%. Studied staphylococci showed no resistance to gentamicin and amoxicillin/clavulanic acid.

Table 5 presents the determined MIC_{90} values to 9 of the tested chemotherapeutics. The highest MIC_{90}, 128 µg/mL and 2 µg/mL, were obtained for tetracycline and lincomycin, respectively. The lowest MIC of 0.001 µg/mL were found out for rifampicin and ciprofloxacin. With regard to oxacillin, MIC_{90} was 0.125 µg/mL; for trimethoprim/sulfamethoxazole it was 1.5 µg/mL; and for ampicillin, cephalothin and cefoxitin it was 1 µg/mL.

Resistance to beta-lactams, lincomycin and tetracycline was present in 15.4% of multi-resistant coagulase-negative staphylococci. The multi-resistance patterns in 21.9% of coagulase-positive staphylococci referred to beta-lactams and rifampicin.

The genetic analysis of strains showed that 10.2% of coagulase-negative staphylococci belonged to *Staphylococcus epidermidis* species, whereas 31.2% of coagulase-positive staphylococci were identified as *Staphylococcus aureus*. Figure 1 presents amplification plots and Cт values obtained from genetic identification of staphylococci. Positive results have C_T values < 34.

Discussion

In this survey, the proportion of staphylococcal isolates from bovine mastitis was 40.5%, coming second after isolates from *Streptococcus* spp. The major part of staphylococcal isolates from milk samples was coagulase-negative (71%), whereas the share of *Staphylococcus aureus* was 31.2% from the group of coagulase-positive strains. Seventy-seven coagulase-negative staphylococcal strains (98.7%) and 25 coagulase-positive strains (78.1%) were associated with subclinical mastitis.

A number of authors have affirmed the increasing role of coagulase-negative staphylococci in the etiology of mastitis in Europe (Tenhagen *et al*., 2009;

Table 2. Surveyed farms, cases with subclinical mastitis, samples submitted to microbiological examination and results

Farm	Total number of cows	Number of cows with clinical mastitis	Number of milk quarters tested with KMT	Of them ++++	Total number of samples for microbiological examination	Number of samples with positive microbiological finding	Positive for *Streptococcus* spp.	Positive for *Staphylococcus* spp spp.	Other species
Trem	280	2	1 112	28	28	22	12	3	7
Kamburovo	114	10	416	23	33	24	8	16	0
Mengishevo	350	10	1360	93	103	67	61	5	1
Karapelit	500	3	1988	75	78	64	30	34	0
Stara Zagora	60	1	236	30	31	26	15	11	0
Popovitsa	96 out of 2500	1	380	34	35	29	10	19	0
Harmanli	61	6	-	-	6	4	2	2	0
Borets	270	1	1076	29	30	36	13	20	3
Total	1731	34	6568	312	346	272	151	110	11

Table 3. Species distribution of microbial isolates from clinical and subclinical bovine mastitis by farms

Farm	Examined samples	Clinical / sub-clinical mastitis	Total number of isolates	Including from clinical / subclinical mastitis	*Streptococcus* spp.		*Staphylococcus* spp.		Other species
					number	species	number	species	
Trem	**30**	2 / 28	**22**	2 / 20	**12**	*S.agalactiae-6* *S.dysgalactiae-4* *S.uberis* - 2	**3**	*S.aureu-2* *CNS-1*	*T.pyogenes–3* *Pasteurella spp.–1* *E.coli-3*
Kamburovo	**33**	10 / 23	**24**	8 / 16	**8**	*S.agalactiae-4* *S.uberis-4*	**16**	*S.aureus-11* *CNS-5*	
Mengishevo	**103**	10 / 93	**67**	10 / 57	**61**	*S.agalactiae-42,* *S.dysgalactiae-7* *S.uberis-12*	**5**	*CNS-5*	*Klebsiella spp.-1*
Karapelit	**78**	3 / 75	**64**	3 / 61	**30**	*S.agalactiae-7* *S.dysgalactiae-8* *S.uberis - 15*	**34**	*S.aureus-5* *CNS-29*	
Stara Zagora	**31**	1 / 30	**26**	1 / 25	**15**	*S.dysgalactiae-9* *S.uberis - 6*	**11**	*S.aureus-3* *CNS-8*	
Popovitsa	**35**	1 / 34	**29**	1 / 28	**10**	*S.dysgalactiae-5* *S.uberis-5*	**19**	*S.aureus-1* *CNS-18*	
Harmanli	**6**	6	**4**	4	**2**	*S.dysgalactiae-2*	**2**	*S.aureus-2*	
Borets	**30**	1 / 29	**36**	1 / 35	**13**	*S.agalactiae-2* *S.dysgalactiae-3* *S.uberis-8*	**20**	*S.aureus-8* *CNS-12*	*T.pyogenes–1* *Acinetobacter spp–1* *Nocardia spp.-1*
Total	**346**	34 / 312	**272**	30 / 242	**151**	*S.agalactiae* – **61** (1 *S.dysgalactiae* – **38** *S.uberis* – **52**	**110**	*S.aureus* – **32** CNS – **78**	**11** *T.pyogenes–3* *Pasteurella spp.–1* *E.coli–3* *Acinetobacter spp.–1* *Nocardia spp.–1*

Table 4. Percentage of staphylococci resistant to 11 chemotherapeutic drugs with confidence limits

Chemotherapeutic drugs	Coagulase-negative staphylococci (n=78)	*Staphylococcus aureus* (n=32)	Total number of staphylococci (n-110)	Confidence limits (CL)
Ampicillin	13 (16.7%)	14 (43.7%)	27 (24.5%)	17.8÷32.8
Amoxicillin/clavulanic acid	-	-	-	
Oxacillin	12 (15.4%)	3 (9.4%)	15 (13.6%)	7.8÷20.6
Cephalothin	1 (1.3%)	-	1 (0.9)	0÷3.5
Cefoxitin	3 (3.8 %)	3 (9.4%)	6 (5.4%)	1.9÷10.3
Lincomycin	50 (64.1%)	5 (15.6%)	55 (50%)	40.7÷59.3
Gentamicin	-	-	-	
Tetracycline	32 (41.0%)	9 (28.1%)	41 (37.3%)	28.5÷46.5
Rifampicin	1 (1.3%)	-	1 (0.9%)	0÷3.5
Ciprofloxacin	2 (2.6%)	-	2 (1.8%)	0.1÷5.1
Trimethoprim/sulphamethoxazole	8 (10.2%)		8 (7.3%)	3.1÷12.8

Table 5. MICs of 9 chemotherapeutic drugs for staphylococcal isolates from subclinical and clinical bovine mastitis (n = 110)

MIC μg/mL																		
Chemotherapeutic drugs	MIC_{90}	0.001	0.125	0.5	0.75	1	1.5	2	3	4	6	8	16	32	64	96	128	192
Ampicillin	1.0		85	2*	5	8	2	4	1	2					1			
Oxacillin	0.125		98	6*		2	1	3*										
Cephalothin	1.0			32	30	20		27			1*							
Cefoxitin	1.0					54		50		2	*	1	1	2				
Lincomycin	2.0			5	45	5		55*										
Tetracycline	128								*		69			1	9	1	4	26
Rifampicin	0.001	109	*								1							
Ciprofloxacin	0.001	108					*							2				
Trimethoprim/ sulphamethoxazole	1.5					30	40	32		3		1*		4				

Legend: MIC thresholds are marked with asterisks

Persson *et al.*, 2011; Piessens *et al.*, 2011), as well as in other regions in the world (Mekonnen *et al.*, 2017; Mpatswenumugabo *et al.*, 2017). In Belgium, Piessens *et al.* (2011) have discussed the thesis that the spread of coagulase-negative staphylococci in mastitis may be associated with the fact that they are human skin commensal organisms that may spread on animals due to the lack of proper hygienic measures. Zadoks *et al.* (2011) and Ndahetuye *et al.* (2019) have emphasized on the fact that the involvement of *Staphylococcus aureus* in the etiology of subclinical mastitis was rather related to chronic and persisting infections.

The genotyping of coagulase-negative staphylococci demonstrated that the commonest species were *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, and *S. xylosus* (Capurro *et al.*, 2009; Perry *et al.*, 2010; Persson *et al.*, 2011; Duse *et al.*, 2021). In Germany, Luthje *et al.* (2006) have reported a different species distribution of coagulase-negative staphylococci causing bovine subclinical mastitis, with the highest prevalence of *Staphylococcus chromogenes* (33.2%), followed by *Staphylococcus simulans* (23.2%) and *Staphylococcus epidermidis* (11.7%). In Rwanda, Ndahetuye *et al.* (2019) have found out that among coagulase-negative staphylococcal agents of subclinical mastitis, *Staphylococcus epidermidis* (38.2%) was more commonly isolated than *Staphylococcus sciuri* (19.5%) and *Staphylococcus chromogenes* (9.8%). On the other hand, Ruegg (2020) has commented that streptococci were the leading agents of bovine clinical mastitis,

Table 6. Resistance patterns of multiresistant staphylococci isolated from bovine mastitis (n-110)

Resistance patterns	Number of resistant strains	Resistant staphylococci (%) and confidence limits (CL)
	Coagulase-negative staphylococci (n=78)	
Ox, Amp, L,T	6	7.7% (2.8÷14.5)
Ox, Amp, Fox, L	2	2.6% (0.3÷7.2)
Ox, Amp, L	2	2.6% (0÷0.3)
Ox, Amp, Ceph, L	1	1.3% (0÷4.9)
Ox, Amp, AMC, Ceph, Fox, L, T	1	1.3% (0÷4.9)
	Coagulase-positive staphylococci (n=32)	
L, RIF	4	12.5% (3.5÷26.1)
Ox, Amp, Ceph, RIF	3	9.4% (1.9÷21.7)

Legend: Ox – oxacillin, Amp-ampicillin, AMC – amoxicillin/clavulanic acid, Ceph – cephalothin, Fox – cefoxitin, L – lincomycin, T – tetracycline, RIF – rifampicin.

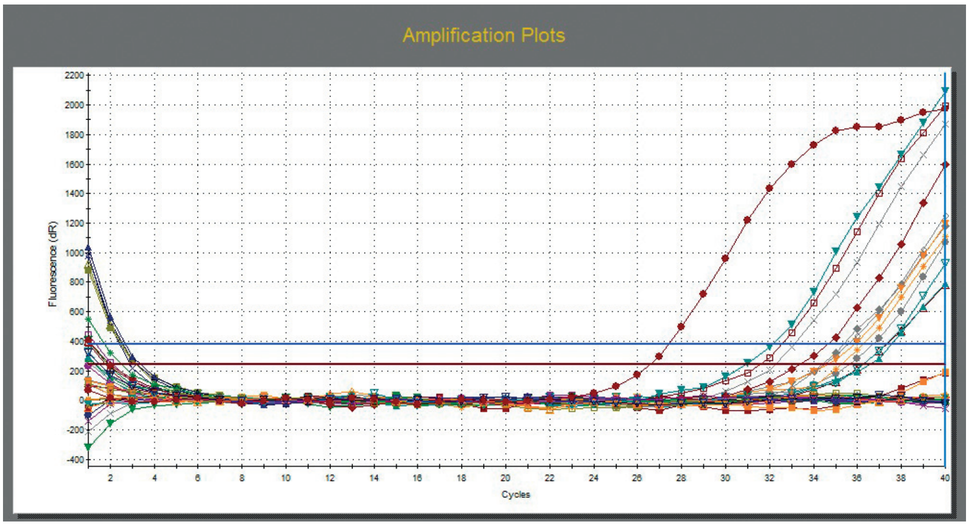

Fig. 1. Amplification plots of *Staphylococcus aureus*

followed by coliform bacteria and *Staphylococcus aureus*. Neelam *et al.* (2022) have also determined *Staphylococcus aureus* as the leading etiological agent of bovine mastitis in India: in 79.7% of cases as seen from genetic analysis of staphylococci isolated from milk samples, whereas only 10.9% were described as belonging to coagulase-negative staphylococci. Also in India, Mahanti *et al.* (2020) have proved the presence of *Staphylococcus aureus* in 21% of milk samples from healthy cows and cows with clinical and subclinical mastitis, tested by phenotype and genetic methods.

From a comparative point of view, the number of genetically determined coagulase-negative *Staphylococcus epidermidis* were encountered at a lower rate (10.2%), comparable with that reported by Luthje *et al.* in Germany (11.8%). Regarding the prevalence of coagulase-positive staphylococci, genetic studies classified 31.2 % of the tested strains as *Staphylococcus aureus*, with a rate higher than those from data of Mahanti *et al.*, and far lower than those reported by Neelam *et al.* in India.

In Poland, Gagielski *et al.* (2014) have discussed the hypothesis that penicillin-resistant staphylococci were largely involved in bovine mastitis etiology (41%). They also underlined that fact that all *Staphylococcus aureus* isolates were sensitive to cefoxitin, gentamicin, doxycycline, ciprofloxacin and trimethoprim/sulfamethoxazole and expressed an intermediate sensitivity to clindamycin. In their view, the monitoring of antimicrobial resistance of *Staphylococcus aureus* strains in dairy cattle farms is an important part of mastitis control and objective therapeutic control.

In Germany, Luthje *et al.* (2006) observed a broader prevalence of ampicillin-resistant coagulase-negative staphylococci (18.1%), as well as of those resistant to pirlimycin from the lincosamides group (6.4%). In Brazil, Olivera *et al.* (2012) have reported a higher prevalence rate to tetracycline (19%) among *Staphylococcus aureus* isolates from bovine subclinical and clinical mastitis. Again in Brazil, Zuniga *et al.*

(2020) have discussed the high rate of coagulase-negative staphylococci, resistant to amoxicillin and ampicillin (59.7%) causing subclinical mastitis in cattle. With regard to gentamicin and oxacillin, the authors recorded a high sensitivity, 95% and 86%. The MIC_{90} values for aminopenicillins exceeded 8 µg/mL.

Our results demonstrated a lower spread of staphylococci resistant to aminopenicillins (24.5%), compared with the data of Zuniga *et al*. from Brazil, with a higher percentage of resistant coagulase-positive strains (43.7%). On the other side, the obtained MIC_{90} values for aminopenicillins were below 1 µg/mL. In India, Mahanti *et al.* (2020) have reported a higher prevalence of *Staphylococcus aureus* resistant to beta-lactam chemotherapeutics ampicillin (71.4%), cefoxitin (42.9%) and amoxicillin/clavulanic acid (38.1%). The authors commented that multi-resistance patterns of methicillin-resistant strains included beta-lactams, tetracyclines and aminoglycosides. In Sweden, possibly due to the antibiotic restriction in livestock husbandry, Duse *et al*. (2021) have reported a high sensitivity of clinical mastitis *Staphylococcus aureus* isolates to chemotherapeutics, except for penicillin-resistant coagulase-negative staphylococci (30.4%).

Also, higher rates of resistance to lincomycin and tetracycline have been reported (50%, 37.3%) compared with data from Germany published by Luthje *et al*. and from Brazil reported by Zuniga *et al*. In India, Neelam *et al*. have also established higher percentages of *Staphylococcus aureus* milk isolates, resistant to lincomycin (49.09%) and oxytetracycline (98.18%). These rates were discussed in the light of the more common use of antimicrobial drugs for bovine mastitis therapy in the country. Our results for microbial resistance to lincomycin and tetracycline showed higher rates in coagulase-negative staphylococci: 64.1% and 41.0%, respectively.

Our data referring to the prevalence of staphylococci sensitive to oxacillin and gentamicin (86.4%, 100%) were similar to the results of Zuniga *et al*. from Brazil.

Conclusion

The information from the present study provides evidence about the higher prevalence of coagulase-negative staphylococci in the etiology of bovine mastitis in Bulgaria and increased rates of resistance among staphylococcal isolates to lincomycin and tetracycline. Multi-resistant patterns including ampicillin, oxacillin, lincomycin and tetracycline were demonstrated in 7.7% a higher prevalence in resistant coagulase-negative staphylococci, whereas 12.5% of *Staphylococcus aureus* strains had multi-resistance patterns including lincomycin and rifampicin.

The monitoring of bacterial antimicrobial resistance, including in agents causing mastitis in animals is recommended by the World Health Organization and the World Organisation for Animal Health to provide valuable information about the trends in therapeutic approaches, namely antibiotic drugs use.

In Bulgaria, beta-lactams, aminopenicillins extended spectrum semi-synthetic penicillins e.g. cloxacillin, and cephalosporins, most commonly cephalothin, are among the drugs most frequently recommended for treatment of bovine mastitis. Intramammary infusions containing combinations of lincomycin and gentamicin, tetracycline, neomycin and bacitracin are also used. In this two-year survey, the demonstrated higher prevalence of lincomycin-resistant and tetracycline-resistant staphylococcal isolates from milk samples is probably a specific feature that should be considered in designing future national-scale studies in this field.

Acknowledgements

This work was supported by a grant from Ministry of Education, Republic of Bulgaria no.12/2020.

References

1. Abebe R, Hatiya H, Abera M, Megersa B & Asmare K (2016) Bovine mastitis: Prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Veterinary Research, 12: 270
2. Capurro A, Artursson K, Persson Waller K, Bengtsson B, Ericsoon Unnerstad H & Aspan A (2009) Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species –level identification of coagulase- negative staphylococci isolated from cases of bovine mastitis. Veterinary Microbiology, 134: 327-333
3. Duse A, Persson-Waller K & Pedersen K (2021) Microbial aetiology, antibiotic susceptibility and pathogen-specific risk factors for udder pathogens from clinical mastitis in dairy cows. Animals, 11, 2113, doi.org/10.3390/ani11072113
4. EUCAST (2021) European Committee on Antimicrobial Susceptibility Testing, Clinical breakpoints-breakpoints and guidance, https://www.eucast.org/clinical_breakpoints (23 march 2022 date last accessed)
5. Gagielski T, Puacz E, Lisowski A, Siedlecki P, Dudziak W, Miedzobrodzki J& Krukowski H (2014) Short communication: antimicrobial susceptibility profiling and genotyping of *Staphylococcus aureus* isolated from bovine mastitis in Poland. Journal of Dairy Science, 97: 6122-6128
6. Léon-Galvan MF, Barboza-Corona JE, Lechuga-Arana AA, Valencia-Posadas M, Aguayo DD, Cedillo-Palaez C, Martinez –Ortega EA & Gutierrez-Chavez AJ (2015) Detection and sensitivity to antibiotics and bacteriocins of pathogens isolated from bovine mastitis in family dairy herds of Central Mexico. BioMed Research, 615153
7. Livermore D & Brown D (2001) Detection of beta-lactamase-mediated resistance. Journal of Antimicrobial Chemotherapy, 48, Suppl 1: 59-64
8. Luthje P& Schwarz S (2006) Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide- lincosamide resistance phenotypes and genotypes. Journal of Antimicrobial Chemotherapy, 57: 966-969
9. 9. Mader R, Damborg P, Amat JP, Bengtsson B, Bourély C, Broens EM, Busani L, Crespo P, Filippitzi ME& Fitzgerald W (2021) Building the European Antimicrobial Resistance Surveillance network in veterinary medicine (EARS-Vet). Eurosurveillance: 26
10. Mahanti A, Siddhartha Narayan Joardar, Samiran Bandyopad-

hyay, Jaydeep Banerjee, Sarbaswarup Ghosh, Kunal Batabyal, Tapas Kumar Sad, Tapan Kumar Dutta & Indranil Samanta (2020) Characterization of methicillin- resistant and enterotoxins producing *Staphylococcus aureus* in bovine milk in India. Journal of Agriculture and Food Research, 2, 100017, doi. org/101016/j.jafr.2019. 100017
11. Mekonnen SA, Koop G, Melkie ST, Getahun CD, Hogeveen H & Lam TJGM (2017) Prevalence of subclinical mastitis and associated risk factors at cow and herd level in dairy farms in North-West Ethiopia. Preventive Veterinary Medicine, 145: 23-5877
12. Mpatswenumugabo JP, Bebora LS, Gitao GC, Mobegi VA, Iraguha B, Kamana O, Shumbusho B & Mestorino Nora (2017) Prevalence of subclinical mastitis and distribution of pathogens in dairy farms of Rubavu and Nyabihu districts, Rwanda. Journal of Veterinary Medicine, 8456713, doi: 10.1155/2017/8456713
13. Neelam VK, Jain Mahavir Singh, Vinay G. Joshi, Rajesh Chhabra, Kuldeep Singh & Rana YS (2022) Virulence and antimicrobial resistance gene profiles of *Staphylococcus aureus* associated with clinical mastitis in cattle. PLOS One, doi. org/10.1371/journa.pone.0264762
14. Ndahetuye JB, Persson Y, Nyman Ann-Kristin Tukei M, Ongol MP& Båge R (2019) Etiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda. Tropical Animal Health and Production, 51: 2037-2044
15. Oliveira L, Langoni H, Hulland C& Ruegg PL (2012) Minimum inhibitory concentration of *Staphylococcus aureus* recovered from clinical and subclinical cases of bovine mastitis. Journal of Dairy Science, 95:1913-1920
16. Persson Y, Nyman A & Grönlund- Andersson U (2011) Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Veterinaria Scandinavica, 53: 3
17. Perry J, Midlleton JR, Dufour S, Scholl D, Calloway C, Anderson S& Dohoo I (2010) Association of coagulase-negative staphylococcal species and milk somatic cell count of cows from the Canadian national cohort of dairy farms. MMC Annual Meeting Procedures, Albuquerque, New Mexico: 204-205
18. Piessens V, Van Coillie E, Verbist B, Supré K, Braem G, Van Nuffel A, De Vuyst L, Heyndrickx M & De Vliegher S (2011) Distribution of coagulase-negative *Staphylococcus* spp. from milk and environment of dairy cows differs between herds. Journal of Dairy Science, 94 (6): 2933-2944
19. Ruegg PL (2018) Making of antibiotic treatment decisions for clinical mastitis. Veterinary Clinical Nort American Food and Animal Practice, 34: 413-425
20. Sakwinska OD, Morisset J, Madec JY, Waldvogel A, Moreillon P& Haenni M (2011) Link between genotype and antimicrobial resistance in bovine-mastitis *Staphylococcus aureus* strains, determined by comparing Swiss and French isolates from the Rhóne Valley. Applied Environmental Microbiology, 77: 3428-3432
21. Salvador RT, Soliven RL, Balagan EJY, Abes NS, Gutierrez CA & Mingala CN (2014) Evaluation of portable somatic cell counter in the diagnosis of bubaline subclinical mastitis. Thai Journal of Agricultural Science, 74 (4): 205-209
22. Schnitt A & Tenhagen Bernd-Alois (2020) Risk factors for the occurrence of methicillin-resistant *Staphylococcus aureus* in dairy herds: An update. Foodborne Pathogens and Disease, 17 (10), doi: 10.1089/fdp.2019.2638
23. Silveira-Filho VM, Luz IS, Campos APF, Silva WM, Barros MPS, Medeiros E, Freitas MFL, Mota RA, Sena MJ& Leal-Balbino TC (2014) Antibiotic resistance and molecular analysis of *Staphylococcus aureus* isolated from cow's milk and dairy products in Northeast Brazil. Journal of Food Protection, 77: 583-591
24. Tenhagen BA, Hansen I, Reinecke A & Heuwieser W (2009) Prevalence of pathogens in milk smples of dairy cows with clinical mastitis and in heifers at first parturition. Journal of Dairy Research, 76: 179-187
25. Vakkamäki J, Taponen S, Heikkilä AM & Pyörälä S (2017) Bacteriological etiology and treatment of mastitis in Finnish dairy herds. Acta Veterinaria Scandinavica, 59: 33
26. Zadoks RN, Middleton JR, McDougall S, Katholm K& Schukken YH (2011) Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Journal Mammary Gland Biology Neoplasia, 16: 357-372
27. Zuniga E, Benites NR, Santana da Hora A, Mello PL, Laes MA, Ribeiro de Olivera LA, Brandäo PE, Oliveira de Souza Silva S, Taniwaki SA & Melville PA(2020) Expression of genes encoding resistance in *Staphylococcus* spp., isolated from bovine subclinical mastitis in Brazil. Journal of Infection in Developing Countries, 14 (7): 772-780

Received 25 October 2022
Accepted 9 December 2022