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Original article

Phenotypical and genotypical antimicrobial resistance of coagulase-negative *staphylococci* isolated from cow mastitis

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Abstract

The objectives of this study were to determine the prevalence and antimicrobial resistance of coagulase-negative staphylococci (CNS) isolated from dairy cows with subclinical mastitis. Antimicrobial resistance in staphylococci were evaluated by breakpoint values specific to the species (EUCAST). The presence of resistance-encoding genes was detected by multiplex PCR. A total of 191 CNS isolates were obtained. The CNS isolates were typically resistant to penicillin (67.4%), tetracycline (18.9%), and erythromycin (13.7%). CNS isolates (78.0%) were resistant to at least one antimicrobial compound, and 22.0% were multiresistant. The multiresistant isolates were predominantly *Staphylococcus chromogenes* (28.6%), *Staphylococcus warneri* (19%) and *Staphylococcus haemolyticus* (14.3%). According to MIC pattern data, multiresistant isolates showed the highest resistance ($p<0.05$) rates to penicillin (85.7%), tetracycline (66.7%), and erythromycin (48.2%), but all of them were sensitive to daptomycin, oxacillin, qiunupristin/dalfopristin, and vancomycin. *S. chrom*ogenes (9.5%), *S. haemolyticus* (4.8%), and *S. capitis ss capitis* (2.4%) strains were resistant to methicillin; their resistance to oxacillin and penicillin was more than 8 mg/l. A high rate of resistance to penicillin was linked to a *bla*Z gene found in 66.6% of the isolated multiresistant CNS strains. Resistance to tetracycline via the *tet*K (38.1%) gene and penicillin via the *mec*A (23.8%) gene were detected less frequently. Gene *msr*AB was responsible for macrolides and lincosamides resistance and detected in 28.6% of the CNS isolates. Antimicrobial resistance genes were identified more frequently in *S. epidermidis*, *S. chromogenes*, and *S. warneri*.

Key words: coagulase-negative staphylococci, mastitis cows, antimicrobial resistance, gene

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Introduction

Mastitis, inflammation of the mammary glands, is usually caused by microbial infection. Mastitis pathogens have been previously studied in Lithuania and different causative agents were identified. The most frequent causative agents of mastitis are streptococci (5.43 – 20.35%), coagulase-negative staphylococci (CNS) (2.86 – 58.15%), and enterobacteria (8.47%); *S. aureus* alone causes 19.97 – 65.0% of mastitis cases (Klimiene et al. 2011). Nowadays, CNS are of great interest in veterinary medicine because they are currently considered emerging pathogens of bovine mastitis. Although CNS are not as pathogenic as other principal mastitis pathogens and CNS infection is mostly subclinical, CNS can cause persistent infections, which result in increased milk somatic cell count and decreased milk quality. Prevalent CNS species vary according to the geographical region under scrutiny (Soares L. C. 2012, Sztachanska et al. 2016).

Mastitis is one of the major causes of antibiotic use in dairy cows. There is a variety of antimicrobials that are used for mastitis prevention and treatment; therefore, antimicrobial resistance is expected. Among the antimicrobial agents approved for use in bovine mastitis, β-lactams, such as penicillins and cephalosporins, play a key role. Resistance to β-lactams in staphylococci is mediated by either β-lactamases encoded by the *bla*Z gene or the *mec*A-encoded alternative penicillin binding protein, PBP2a, which shows a reduced binding to the β-lactam antibiotics currently available for mastitis therapy (Aarestrup et al. 2006). Antibiotic-resistant udder pathogens are spread worldwide, with regionally different resistance patterns. The antimicrobial resistance of mastitis pathogens has received much interest over the past few years. Carriage of antimicrobial resistance genes by CNS species in cattle may also be relevant because it potentially poses a human health hazard; this can happen both through the lateral transfer of resistance genes between staphylococcal species and through the direct transmission of resistant pathogens (Walther and Perreten, 2007). Humans and dairy cattle may share CNS strains, implying that multidrug-resistant, bovine staphylococci might be zoonotic pathogens. It is dificult to demonstrate the direction of interspecies transmission, but it has been suggested that CNS are more likely to spread from humans to dairy cattle than vice versa (Thorberg et al. 2009).

The aim of this study was to analyze the CNS of the subclinical mastitis cow and determine the resistance to antimicrobial agents, particularly phenotypic and genotypic resistance.

Materials and Methods

Place and samples

In 2014 samples were collected from bovine dairy farms in Lithuania. A total of 450 animals were evaluated by California Mastitis Test and 214 cows were positive for subclinical mastitis. Individual mammary quarter milk samples were aseptically collected into sterile vials immediately before milking, after discarding the first three milking streams. The milk samples were transported to laboratory during 2 hours for furher investigation.

Isolation and identification of *Staphylococcus* spp.

Clinical material was inoculated onto 5% Sheep Blood Agar, Mannitol Salt Agar (Liofilchem, Italy) supplemented with 4 mg/L cefoxitin (Sigma-Aldrich) and Brilliance MRSA 2 Agar (Oxoid, Thermo Fisher, UK). Presumptive identification of *Staphylococcus* genus was based on the growth and morphology characteristics, catalase production, gram-staining and susceptibility to furazolidone. Species identification was performed only for the isolates that grew on Mannitol Salt Agar supplemented with 4 mg/L cefoxitin and/or Brilliance MRSA 2 Agar. Single colonies were taken from the agar surface and re-cultivated on the Mannitol Salt Agar supplemented with cefoxitin and Brilliance MRSA 2 Agar with the aim to obtain pure cultures. Presumptive species identification was based on pigment and coagulase production, presence of protein A and clumping factor as well as on biochemical properties detected by using RapID Staph Plus (Thermo Scientific) identification system. In uncertain identification cases Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) analysis (VITEK MS, Biomerieux, France) was used as described previously (Dubois D. et al. 2012).

Phenotypic antimicrobial tests

The inoculum was obtained from overnight broth cultures and adjusted to achieve approximately 5×10^5 CFU/ml considering a turbidity equivalent to a 0.5 McFarland standard (CLSI, 2010). Disk diffusion test was employed to determine the susceptibility of penicillin (10UI), gentamicin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), cefoxitin (30 μg) and trimethoprim-sulfamethoxazole (1,25/23,75 μg), (SENSIFAR-CEFAR® agents. Strains resistant to above two antimicrobial classes

Table 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5' – 3')	Size, bp and T(°C)	Target gene	Source
mecA1 mecA2	GGGATCATAGCGTCATTATTC AACGATTGTGACACGATAGCC	527 (61)	*mecA*	Anonymous, 2008
mecC1 mecC2	GCTCCTAATGCTAATGCA TAAGCAATAATGACTACC	204 (50)	*mec*LGA251	Cuny et al. 2011
16S1 16S2	GTGCCAGCAGCCGCGGTAA AGACCCGGGAACGTATTCAC	886 (61)	16S staph	Anonymous, 2008
blaZ1 blaZ2	CAGTTCACATGCCAAAGAG TACACTCTTGGCGGTTTC	772 (50)	*blaZ*	Schnellmann et al. 2006
tetM1 tetM2	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	656 (45)	*tet*(M)	Aarestrup et al. 2000
tetK1 tetK2	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	718 (55)	*tet*(K)	Aarestrup et al. 2000
aac6-aph2F aac6-aph2R	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	348 (61)	*aac(6')-Ie-aph(2'')-Ia*	Perreten et al. 2005
aph3-IIF aph3-IIR	CCGCTGCGTAAAAGATAC GTCATACCACTTGTCCGC	609 (57)	*aph(3')-IIIa*	Perreten et al. 2005
dftrG1 dfrG2	TTTCTTTGATTGCTGCGATG AACGCACCCGTTAACTCAAT	501 (51)	*DfrG*	Couto et al. 2001
dfrK1 dfrK2	GCTGCGATGGATAAGAACAG GGACGATTTCACAACCATTAAAGC	214 (50)	*Dfr*K	Kadlec et al. 2010
ermA1 ermA2	AAGCGGTAAAACCCCTCTGAG TCAAAGCCTGTCGGAATTGG	442 (53)	*erm*(A)	Jensen et al. 2002
ermC1 ermC2	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	295 (48)	*erm*(C)	Jensen et al. 2002
msrAB1 msrAB2	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	350 (55)	*msr*A/B	Thumu et al. 2012

were considered multiresistant. *Staphylococcus aureus* ATCC25923 was used as positive control.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the broth microdilution method. Sensititre® plates and the ARIS 2X automated system (Thermo Scientific) were used with the following antimicrobials: oxacillin, penicillin, clindamycin, erythromycin, gentamicin, tetracycline, daptomycin, ciprofloxacin, levofloxacin, linezolid, quinupristin/dalfopristin, vancomycin, co-trimoxazole and rifampin. Interpretation of results was carried-out using manufacturers software (SWIN®) adapted to clinical breakpoints of European Committee on antimicrobial susceptibility testing (EUCAST). The quality control strain *S. aureus* ATCC 29213 was included in each assay for validation purposes.

DNA extraction

DNA material for molecular testing was obtained after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance (Anonymous, 2008) with slight modifications. Briefly, a loopful of colonies were taken from the surface of Mueller Hinton Agar and transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min. Then the supernatant was discarded and the pellet was re-suspended in Tris-EDTA (TE) buffer. The suspension was heated using a thermomixer at 100°C degrees for 10 minutes. Boiled suspension was transferred directly on ice and diluted by 1:10 in TE.

PCR assay for antimicrobial genes

Detection of genes encoding antimicrobial resistance *(mecA, mecC, blaZ, tet*(K), *tet*(M), *erm*(A),

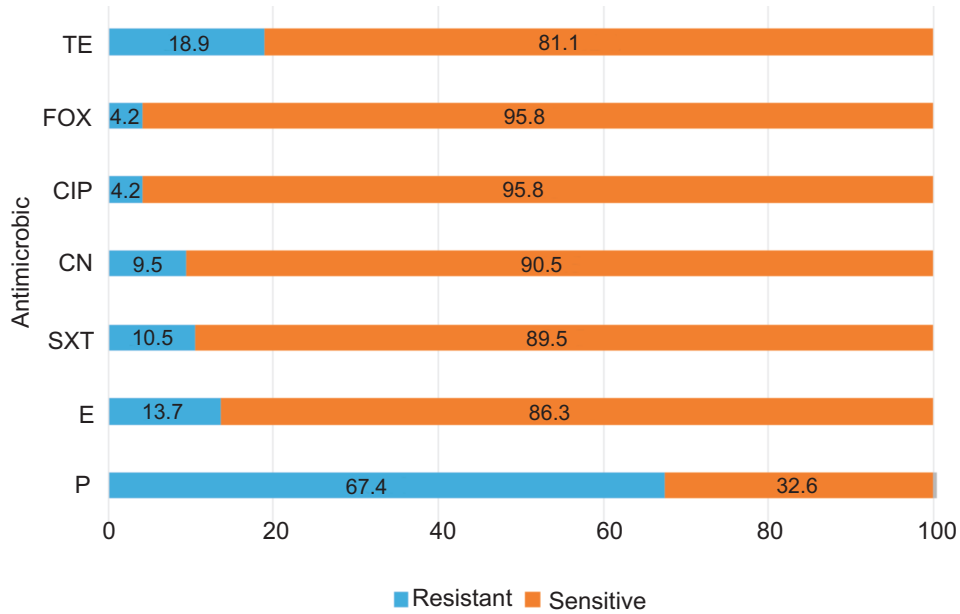

Fig. 1. *Staphylococcus* species resistant to antimicrobic drugs spread in cow mastitis, (n=95)
P.S. P – penicillin, E – erythromycin, SXT – sulfamethoxazole/trimethoprim, CN – gentamicin, CIP – ciprofloxacin, FOX – cefoxitin, TE – tetracycline.

Table 2. Combination of antimicrobials in multiresistant CNS strains (n=42)

Number of isolates	Antimicrobic						
	Penicillin	Erythromycin	SXT	Gentamicin	Ciprofloxacin	Cefoxitin	Tetracycline
2	■	■	■				
2	■				■		■
4		■	■				■
14	■	■					■
2	■		■	■	■		■
2	■	■	■				■
2	■		■	■			
4	■		■				■
3	■					■	■
2	■	■		■			
3	■		■	■		■	■
2	■	■	■	■	■	■	■

P.S. SXT – sulfamethoxazole/trimethoprim

erm(C), *msr*AB, *aac(6')-Ie-aph(2'')-Ia, aph(3')-IIIa*, *dfrG* and *dfrK*) was performed by PCR. Annealing temperatures and oligonucleotides used are presented in Table 1.

Statistical analysis

Statistical analysis was performed using „R 1.8.1" package (http://www.r-project.org/ webcite). Comparison between categorical variables was calculated by chi-square and Fisher's exact test. Results were considered statistically significant if $p<0.05$.

Results

Two hundred and fifty-eight staphylococcus isolates were obtained from cows with subclinical masti-

tis; 191 isolates were confirmed as CNS. Most of the CNS isolates were susceptible to ciprofloxacin (95.8%) and sulphametoxasol/trimethoprim ($p<0.05$). Resistance to penicillin was observed in 67.4% of the CNS isolates, but these isolates were less resistant to tetracycline and erythromycin ($p<0.05$) (Fig. 1).

One hundred and forty-nine CNS isolates (78.01%) were resistant to at least one antimicrobial compound, and 21.9% were considered multiresistant. Multiresistant strains are presented in Table 2.

CNS resistant to penicillin and tetracycline were predominant, and most of these strains were resistant to macrolide (erythromycin) and sulfamethoxazole/trimethoprim (Table 2). Two isolates were resistant to all the antimicrobials used in this study. *S. chromogenes* (28.6%), *S. warneri* (19.0%), and *S. haemolyticus* (14.3%) were the most common species isolated amongst the multiresistant CNS. Other species, such as *S. capitis ss capitis, S. hominis, S. epidermidis,* and *S. xylosus* were isolated at the same frequencies but were less predominant (Table 3).

Table 3. *CNS* species multiresistant to antimicrobic drugs spread in cow mastitis, (n=42).

CNS species	Number of isolates	Percentage
S. chromogenes	12	28.6
S. warneri	8	19.0
S. hemolyticus	6	14.3
S. epidermidis	4	9.5
S. hominis	4	9.5
S. xylosus	4	9.5
S. capitis ss capitis	4	9.5

According to the MIC pattern data, all multiresistant CNS isolates were sensitive to daptomycin, oxacillin, qiunupristin/dalfopristin, and vancomycin, but showed higher resistance to penicillin (85.7%), tetracycline (66.7%), and erythromycin (48.2%). All these data are statistically reliable ($p<0.05$). Two *S. chromogenes* strains, one *S. haemolyticus* strain, and one *S. capitis ss capitis* strain were resistant to methicillin. Their resistance rates to oxacillin and penicillin were more than 8 mg/l.

Phenotypical and antimicrobial resistance encoding gene patterns are presented in Table 5.

In 14 cases, the disk diffusion data were not similar to the MIC values. Nine strains were determined to be resistant to sulfamethoxasole by the disk diffusion method, but this resistance was not confirmed by MIC. The same discrepancy was in 3 cases with tetracycline, 5 with erythromycin, and 2 with gentamycin. Genes encoding antimicrobial resistance were not detected in these cases either; together, these results suggest that the disk diffusion method is more prone to yield false positives. Antimicrobial resistance-encoding genes in CNS isolates were determined by PCR. A *bla*Z gene related to producing β-lactamases was found in 66.6% of the identified CNS strains; Tetracycline resistance-encoding *tet*K (38.1%) and *mec*A (23.8%), which encodes penicillin binding protein, were detected less frequently. The *ms*rAB gene, responsible for macrolide and lincosamide resistance, was detected in 28.6% of CNS isolates. Antimicrobial resistance genes were identified more often in *S. epidermidis*, *S. chromogenes,* and *S. warneri* CNS species.

Discussion

CNS are often associated with clinical or subclinical mastitis in cows, and CNS are isolated quite often. In the past, CNS were considered as part of the opportunistic cow udder skin microflora. Mastitis wasn't difficult to treat, but nowadays it is a serious problem for dairy milk farms. About 10-20% of all udder inflammation cases during the first lactation period are caused by CNS (Pyorala and Taponen 2009). The prevalence of the most common specific pathogen and the range of CNS' biological properties become problematic when trying to keep the epidemiology situation under control (Piessens et al. 2011, Sztachanska et al. 2016). During our study, CNS were isolated in 74.03% of cases of subclinical mastitis. The most prevalent species were *S. chromogenes* (28.6%) and *S. warneri* (19%). *S. chromogenes* is also considered the most common subclinical mastitis pathogen in Belgium, Finland, Sweden, and the USA (Pyorala and Taponen, 2009, Thorberg et al. 2009, Gillespie et al. 2009, Sawant et al. 2009, Supré et al. 2011, Piessens et al. 2012). *S. chromogenes* was more frequently isolated from first-calf period milk; *S. simulans* and *S. epidermidis* were isolated from the milk of older cows. *S. warneri* comprises about 2% of CNS cases (Gillespie et al. 2009), but during our research, *S. warneri* were identified in 19% of the cases of subclinical mastitis.

The indicated staphylococcus isolates have phenotypic resistance to antimicrobials that have been used for a while, such penicillins, macrolides, and tetracycline. This was confirmed by phenotypic and genotypic (PCR) methods. The *bla*Z gene encoding β-lactamases (66.6%) and *mec*A encoding penicillin binding protein (23.8%) were detected in resistant strains. The *mec*A gene contains a mobile chromosomal cassette mec (SCCmec) and is responsible for staphylococcus resistance to methicillin and other β-lactam antimicrobials. Such resistance was confirmed by genetic methods but varies in other

Table 4. MIC distribution (%) *Staphylococcus* spp. strains of cow mastitis, n=42.

Antimicrobial drug	MIC distribution (%). mg/l											
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
Ampicilliną		14.3	47.6	19			9.5	4.8	4.8			
Ceftriaxoneą								76.2	23.8			
Ciprofloxacin				80.9	4.8	14.3						
Clindamycin		57.1	9.5		14.3	19.1						
Daptomycin			85.7	14.3								
Erythromycin			38.1	14.3	4.8		42.8					
Gatifloxaciną					85.7	9.5	4.8					
Gentamyciną						80.9	14.3	4.8				
Levofloxacin			61.9	19	4.8		9.5	4.8				
Linezolid				52.4	23.8	23.8						
Oxacillin+2% NaCl			47.6	14.3	19		4.8	14.3				
Penicillin	9.5	4.8	28.6	28.6	9.5		4.8	14.3				
Quinupristin/dalfopristin		14.3	47.6	19	14.3		4.8					
Rifampin				100								
Tetracycline						33.3	14.3	14.3	38.1			
Trimethoprim/sulfamethoxazole				90.5				9.5				
Vancomycin					100							

P.S. green check – sensitive, blue check- on average sensitive; reed check – resistanse.
[1] – CLSI standart

Table 5. Staphylococcus species, phenotypical resistance and antimicrobial resistance encoding gene pattern.

Staphylococcus species	Antimicrobial resistance, MIC (mg/l)	Genes encoding antimicrobial resistance
S. hominis	P, TE	*bla*Z, *tet*K
S. warneri	P	*bla*Z
S. capitis ss capitis	P	*bla*Z
S. warneri	AMP, E, P	*bla*Z, *msr*AB
S. chromogenes	AMP, AXO, CIP, E, GAT, CN, LEVO, OXA, P, TE, SXT	*bla*Z, *mec*A, *msr*AB, *tet*K, *dfr*G
S. chromogenes	AMP, AXO, CN, OXA, P, TE	*bla*Z, *mec*A, *tet*K
S. warneri	AMP, CN, P	*bla*Z, *aac*(6), dfrG
S. chromogenes	CIP, CLI, GAT, LEVO, P, TE	*bla*Z, *erm*C, *aac*(6)
S. warneri	AMP, CLI, E, P, TE	*msr*AB, *tet*K
S. epidermidis	AMP, E, P, TE	*bla*Z, *mec*A, *msr*AB, *tet*K
S. hominis spp. hominis	AXO, P, TE	*tet*K
S. haemolyticus	P	*bla*Z
S. xylosus	AMP, P, TE	*bla*Z, *tet*K
S. haemolyticus	AMP, AXO, CIP, CLI, E, GAT, CN, LEVO, OXA, P, TE, SXT	*bla*Z, *mec*A, *msr*AB, *aac*(6), *aph*(3), *tet*K, *dfr*G
S. chromogenes	P, TE	*bla*Z
S. haemolyticus	E, P, TE	*bla*Z, *msr*AB
S. capitis ss capitis	CLI, OXA, P, TE	*mec*A
S. chromogenes	CLI, E, TE	–
S. chromogenes	CLI, E, TE	*erm*C
S. epidermidis	E, P	*msr*AB
S.xylosus	AXO, CLI	–

P.S.: P – penicillin, E – erythromycin, SXT – sulfamethoxazole/trimethoprim, CN – gentamicin, CIP – ciprofloxacin, FOX – cefoxitin, TE – tetracycline, AXO – ceftriaxone, CLI – clindamycin, OXA – oxacillin, LEVO – levofloxacin, GAT – gatifloxacin, AMP – ampicillin.

countries. The *bla*Z gene in CNS isolates from mastitis milk were detected in Switzerland (90.7%), Brazil (16.0%), and Poland (8.7%); however, it is consequently agreed that prophylactically using antimicrobials for mastitis treatment makes β-lactam resistance more frequent (Malinowski et al. 2011, Soares et al. 2012, Frey et al. 2013).

According to the veterinary drug registry of The State Food and Veterinary Service of the Republic of Lithuania, β-lactam-class antimicrobials are used most frequently for animal treatment in Lithuania (http://vmvt.lt/node/1161). This study confirmes that intense usage of this kind of antimicrobial inevitably decreases their efficiency and increases microbial resistance to them. Four CNS isolates showed resistance to penicillin and oxacillin with MIC values ≥8 mg/l. Both disk diffusion and MIC data confirmed high resistance to penicillin ($p<0.05$). Two *S. chromogenes* strains expressed *bla*Z, *mec*A, and genes encoding resistance to tetracycline, macrolide, and sulfamethoxazole. Two *S. haemolyticus* isolates were resistant to 7 of the antimicrobials used in this study. Carrying the *mec*A gene and methicillin-resistant CNS isolates could be also associated with other staphylococci, such as *S. haemolyticus*, *S. epidermidis, S. capitis, S. xylosus,* and *S. simulans* (Fessler et al. 2010 http://www.biomedcentral.com/1746-6148/7/6/ – B19, Kot et al. 2012). Eleven percent of staphylococci isolated from cow mastitis in neighboring Poland were resistant to tetracycline, where 47.4% of these CNS isolates were encoded by the *tet*K gene (Kot et al. 2012). The rising incidence of resistance-encoding genes is usually related to long-term usage of penicillin and tetracycline to treat various infections in the veterinary field.

Macrolides, lincosamides, and streptogramins (MLS-class antimicrobials) are widely used for staphylococcus infection treatment in dairy farms. Gram-positive microorganisms have developed three different mechanisms to acquire antimicrobial resistance, and the best known is methylase-influenced protein translation, which is suppressed after antimicrobials bind ribosomes. An antimicrobial efflux-based system encoded by *msr*A and *ms*rAB is also widely known (which removes macrolides and streptogramin B). In our study, two *S. chromogenes* (9.5%) strains showed resistance that was encoded by *erm*C, while *msr*AB genes were encoded more often (28.6%). Other reports indicate more frequent resistance encoded by macrolide genes. In Poland, *erm*A was detected in 14.8%, *erm*B in 11.1%, and *erm*C in 55.5% of cases amongst CNS isolates resistant to macrolides. In our study, other genes encoding resistance to macrolides were not detected, but strain resistance (assessed by MIC) was greater than 4 fg/ml, so macrolide resistance might be associated with other resistance mechanisms.

The use of the combination of trimethoprim and sulfamethoxazole for CNS infection is widely used; it suppresses folate synthesis, which also leads to DNR replication suppression. This leads to effective dihydrofolate reductase *dfr* gene transfer between bacteria strains. This kind of resistance mechanism develops rapidly and widely (Skold 2001). We detected the *dfr*G gene in three (*S. haemolyticus, S. warneri,* and *S. chromogenes*) multiresistant strains, though other strains were detected as phenotypically resistant to macrolide-class antimicrobials.

According to the obtained data, it is necessary to think before using antimicrobials in the lactation and post-lactation periods in dairy farms. Irresponsible usage of antimicrobials leads to antimicrobial treatment failure in hospitals and society.

Conclusion

CNS isolates showed high rates of resistance to penicillins, tetracycline, and macrolides. Resistance to these antimicrobials are linked to the *bla*Z, *mec*A, and *tet*K genes. CNS antimicrobial resistance is increasing in Lithuanian dairy farms, caused by treating animals with penicillins and tetracyclins, which become less efective in subclinical mastitis treatment.

CNS isolates have distinguishingly high resistance rates to antimicrobials. Abundant antimicrobial usage for mastitis treatment leads to the spread of genetic resistance mechanisms among CNS strains. Consiquently, tetracycline- and β-lactam-class antimicrobials are not effective anymore due to the high resistance rates in CNS isolated from cows with subclinical mastitis.

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