FULL PAPER *Bacteriology*

Antibiotic Resistance in *Staphylococcus aureus* Strains Isolated from Cows with Mastitis in Eastern Poland and Analysis of Susceptibility of Resistant Strains to Alternative Nonantibiotic Agents: Lysostaphin, Nisin and Polymyxin B

Piotr SZWEDA[1)]*, Marta SCHIELMANN[1)], Aneta FRANKOWSKA[2)], Barbara KOT[2)] and Magdalena ZALEWSKA[2)]

[1)]*Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, G. Narutowicza 11/12, 80–233 Gdańsk, Poland*

[2)]*Department of Microbiology, Institute of Biology, Siedlce University of Natural Sciences and Humanities, Bolesława Prusa 12, 08–110 Siedlce, Poland*

(Received 8 April 2013/Accepted 28 October 2013/Published online in J-STAGE 11 November 2013)

ABSTRACT. The aim of this study was to analyze the resistance of *Staphylococcus aureus* isolates from bovine mastitis in the eastern part of Poland to a set of 20 antibiotics and three alternative agents: lysostaphin, nisin and polymyxin B. Eighty-six out of 123 examined isolates were susceptible to all 20 tested antibiotics (70%). The highest percentage of resistance was observed in the case of β-lactam antibiotics: amoxicillin (n=22, 17.9%), ampicillin (n=28, 22.8%), penicillin (n=29, 23.6%) and streptomycin (n=13; 10.6%). Twenty-five of the penicillin-resistant strains were found to carry the *blaZ* gene coding for β-lactamases. Two strains were found to be *mecA* positive and a few strains were classified as multidrug resistant (MDR), one of them was simultaneously resistant to six antibiotics. All strains, resistant to at least one antibiotic (n=37) and two control strains, were susceptible to lysostaphin with MIC values of 0.008–0.5 μg/m*l* (susceptibility breakpoint 32 μg/m*l*). Twenty-one (54%) isolates were susceptible to nisin. The MIC value of this agent for 17 (44%) strains was 51.2 μg/m*l* and was not much higher than the susceptibility breakpoint value (32 μg/m*l*). Polymyxin B was able to inhibit the growth of the strains only at a high concentration (32–128 μg/m*l*). The presented results confirmed the observed worldwide problem of spreading antibiotic resistance among staphylococci isolated from bovine mastitis; on the other hand, we have indicated a high level of bactericidal activity of nisin and especially lysostaphin.

KEY WORDS: lysostaphin, mastitis, nisin, polymyxin B, *Staphylococcus aureus*.

doi: 10.1292/jvms.13-0177; *J. Vet. Med. Sci.* 76(3): 355–362, 2014

Bovine mastitis is a disease of major economic importance in the dairy industry worldwide due to loss of milk production and reduction of milk quality as well as an increased usage of drugs and veterinary services. In the United States, the economic loss in the dairy industry due to mastitis is about US $2 billion, and in the United Kingdom, it is £300 million annually, whereas in the Netherlands, the estimated cost varies from €114 to €182/cow per year [12, 13, 37].

Although about 140 species of microorganisms have been identified as etiological agents of bovine mastitis [38], streptococci, coliforms and staphylococci are most often isolated [21, 26, 30, 34]. The mastitis caused by *S. aureus* is characterized by significantly lower cure rates compared with infections caused by other microorganisms. This phenomenon is mainly a result of unusually frequent acquisition of antibiotic resistance mechanisms among this group of bacteria and also their ability to form biofilm (slime) [8]. Biofilm production is considered to be the major reason for recurrence and for the difficulty in eradicating infections of mammary glands [23]. The dramatic spreading of antibiotic-resistant staphylococci and also other groups of microorganisms is caused by unreasonable usage of chemotherapeutics, especially during long-term therapy with the same group of antibiotics and their usage without a prior susceptibility assay of the etiological factor responsible for the infection. More appropriate employment of antibiotics is probably the simplest method to reduce the development of resistance phenomenon. On the other hand, there is an urgent need to look for new antimicrobial agents that are not covered by current existing mechanisms of resistance. The aim of our study was to analyze the resistance of *S. aureus* strains isolated from mastitis milk samples in the eastern part of Poland (the region called Podlasie) to a broad spectrum of antibiotics and also some alternative nonantibiotic agents: lysostaphin, nisin, polymyxin B and lysozyme. The latter compound, which has no anti-staphylococcal activity, was used as a control. Mainly because of O-acetylation in position C-6 of the *N*-acetylmuramic acid (NAM) residue of the peptidoglycan, staphylococci are, in contrast to most G+ bacteria, resistant to lysozyme [2, 6]. Other examined agents, lysostaphin, nisin and polymyxin B, deserve special attention due to their potential application as alternative agents in treatment of staphylococcal infections including bovine mastitis. Although several authors have presented

*CORRESPONDENCE TO: SZWEDA P., Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, G. Narutowicza 11/12, 80–233 Gdańsk, Poland. e-mail: piotr.szweda@wp.pl

Table 1. Sequences of starters used for PCR analysis

No.	Detected gene	Primer sequences	Size of the PCR product (bp)	References
1	*nuc*	nucF: 5′– GCGATAGATGGTGATACGGTT nucR: 5′– AGCCAAGCCTTGACGAACTAAAGC	270	[4]
2	*mecA*	mec1: 5′–AAAATCGATGGTAAAGGTTGG mec2: 5′–AGTTCTGCAGTACCGGATTTGC	533	[24]
3	*blaZ*	blaZ1: 5′–AAGAGATTTGCCTATGCTTC blaZ2: 5′– GCTTGACCACTTTTATCAGC	517	[36]

promising results of examination aimed at analysis of their antimicrobial activity against subclinical and clinical staphylococci isolates [5, 7, 9, 40–42], in our opinion, they are still not given serious enough consideration as effective therapeutic agents for treatment of bovine mastitis and other types of human and animal infectious diseases. In this paper, we show that two of the tested agents, lysostaphin and nisin, have high levels of activity against the strains tested including strains resistant to tested antibiotics.

MATERIALS AND METHODS

Bacterial strains: A total of 123 strains of *S. aureus* isolated in 2007 and 2008 from subclinical bovine mastitis (SCM) milk samples were included in this study. The bacterial strains were identified as *S. aureus* by colonial and microscopic morphology examination and tube tests for coagulase and catalase activity. The strains were additionally tested by using PCR analysis to amplify the part of the *nuc* gene, encoding thermostable nuclease, specific for *S. aureus* [4]. The primer sequences and PCR conditions are presented in Table 1. This part of the analyses was performed during our previous research aimed at analysis of the biofilm production of these strains [32].

Antimicrobial susceptibility testing: Bacteria were grown at 37°C for 18 to 24 hr on a nonselective Luria agar medium (A&A Biotechnology, Gdynia, Poland), and their antimicrobial susceptibility was evaluated using the disk diffusion method on Mueller-Hinton agar (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The following antibacterial agents (Oxoid, Thermo Fisher Scientific Inc., Lenexa, KS, U.S.A.) were tested: penicillin (P; 10 i.u), amoxicillin (AML; 25 μg), amoxicillin with clavulanic acid (AMC; 30 μg), ampicillin (AMP; 10 μg), cephalothin (KF; 30 μg), clindamycin (DA; 2 μg), cloxacillin (OB; 5 μg), cefoperazone (CFP; 75 μg), erythromycin (E; 15 μg), gentamicin (CN; 10 μg), enrofloxacin (ENR; 5 μg), lincomycin (MY; 15 μg), linezolid (LZD; 30 μg), neomycin (N; 30 μg), vancomycin (VA, 30 μg), oxacillin (OX; 1 μg), rifampicin (RD; 5 μg), streptomycin (S; 10 μg), tetracycline (TE; 30 μg) and trimethoprim/sulfamethoxazole (SXT; 25 μg). The inoculated agar plates with discs were left at room temperature for 30 min and then incubated at 35°C for 24 hr. Following the incubation, the diameters of the inhibition zones were measured in millimeters and compared with the ranges suggested by the manufacturer of the disks (Oxoid, Thermo Fisher Scientific Inc.). The strains were classified as resistant, intermediate or susceptible on the basis of the size of the inhibition zone.

MIC assay: The strains that exhibited resistance to at least one tested antibiotic (n=37) and two strains susceptible to all chemotherapeutics were additionally analyzed for resistance to lysostaphin, nisin, polymyxin B and lysozyme (all from Sigma-Aldrich Corp.). The minimal inhibitory concentration (MIC) values of these agents were determined using the broth microdilution method according to the standard protocol of the CLSI with a slight modification proposed by Kusuma and Kokai-Kun [20]. Briefly, twofold dilutions of tested agents were performed in cation-adjusted Mueller-Hinton broth (Sigma-Aldrich Corp.) supplemented with 2% of NaCl and 0.1% of bovine serum albumin (BSA; Sigma-Aldrich Corp.). Wells of a 96-well polystyrene plate were inoculated with 5×10^5 CFU/m*l* of *S. aureus* strains per well diluted from the overnight culture of the bacteria grown on Luria agar. A control comprised of growth of strains with no tested agents was included in each assay. Microtiter plates were incubated at 37°C for 24 hr. Following the incubation, the determination of the MIC values of the tested agents was performed by measuring the absorbance at 531 nm using a Vector^3 microplate reader (PerkinElmer, Inc., Waltham, MA, U.S.A.). The lowest concentration of antibiotic yielding inhibition of growth equal or higher than 90% of growth control was taken as the MIC value. The presence of 0.1% of BSA in each well was necessary to inhibit nonspecific lysostaphin (and probably other agents) adherence to the polystyrene plate, which was previously observed by Climo *et al.* [7]. The MIC assay for each tested strain was done at least three times.

β-lactamase production: The production of β-lactamases, the enzymes responsible for resistance to β-lactam antibiotics, was analyzed using nitrocefin discs according to the manufacturer's instructions (Remel, Thermo Fisher Scientific Inc., Lenexa, KS, U.S.A.). The strains were additionally tested for presence of the *blaZ* gene coding for these enzymes. PCR resulted in amplification of a 517 bp fragment of the *blaZ* gene.

Isolation of DNA: Each isolate was subcultured overnight in 1 m*l* of Luria broth (A&A Biotechnology). After centrifugation (12,000 × *g*, 1 min), DNA was purified from bacterial cells using a Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer's instruction with minor modifications. Namely, 10 μ*l* of lysostaphin (1 U; Sigma-Aldrich Corp.) solution was added

Table 2. The resistance rates of *S. aureus* subclinical mastitis isolates for particular antibiotics

No.	Antibiotic	Number of resistant/intermediately resistant strains	% of resistant/ intermediately resistant strains	Code number of resistant/intermediately resistant isolate
1	P	29/0	23.6/0	1, 5, 9, 11, 29, 34, 38, 39, 42, 54, 70, 71, 72, 74, 75, 78, 83, 84, 86, 93, 94, 95, 101, 102, 103, 104, 105, 112, 113 / -
2	AMP	28/0	22.8/0	1, 5, 9, 11, 29, 34, 38, 39, 42, 54, 70, 71, 72, 74, 75, 78, 83, 84, 86, 93, 94, 95, 102, 103, 104, 105, 112, 113 / -
3	AML	22/1	17.9/0.8	1, 5, 9, 11, 29, 54, 70, 71, 72, 74, 75, 83, 84, 86, 93, 94, 95, 102, 103, 104, 105, 113 / 78
4	S	13/0	10.6/0	6, 11, 27, 66, 76, 84, 88, 93, 94, 95, 101, 116, 119 / -
5	E	3/0	2.4/0	27, 101, 112 / -
6	MY	2/0	1.6/0	27, 101 / -
7	TE	2/0	1.6/0	53, 70 / -
8	N	2/0	1.6/0	27, 101 / -
9	AMC	2/0	1.6/0	1, 11 / -
10	DA	2/0	1.6/0	27, 101 / -
11	SXT	1/0	0.8/0	12 / -
12	CFP	0/5	0/4.1	- / 1, 83, 84, 86, 103, 105
13	OX	0/1	0/0.8	- / 101

P: Penicillin, AMP: Ampicillin, AML: Amoxicillin, S: Streptomycin, E: Erythromycin, MY: Lincomycin, TE: Tetracycline, N: Neomycin; AMC: Amoxicillin with Clavulanic acid, DA: Clindamycin, SXT/trimethoprim/sulfamethoxazole, CFP: Cefoperazone, OX: Oxacillin.

Table 3. The antibiotic resistance profile of *S. aureus* mastitis isolates

No.	Number of antibiotics for resistance/intermediate resistance	Phenotype of resistance	Number of strains
1	1/0	SXT	1
		TE	1
		P	1
		S	5
2	2/0	AMP+P	4
3	2/1	AMP+P/AML	1
4	3/0	AMP+P+AML	11
		AMP+P+E	1
5	3/1	AMP+P+AML/CFP	3
6	4/0	AMP+P+AML+S	4
		AMP+P+AML+TE	1
7	4/1	AMP+P+AML+AMC/CFP	1
		AMP+P+AML+S/CFP	1
8	5/0	AMP+P+AML+AMC+S	1
		DA+E+MY+S+N	1
9	6/1	P+DA+E+MY+S+N/OX	1

P: Penicillin, AMP: Ampicillin, AML: Amoxicillin: S: Streptomycin: E: Erythromycin, MY: Lincomycin: TE: Tetracycline, N: Neomycin: AMC: Amoxicillin with clavulanic acid, DA: Clindamycin, SXT: Trimethoprim/sulfamethoxazole, CFP: Cefoperazone, OX: Oxacillin.

to the cell suspension for enzymatic lysis of *S. aureus* cell wall murein. The mixture was incubated at 37°C for 30 min. Obtained DNA solutions were stored at −20°C until further analysis.

PCR conditions: The amplification reactions were performed using an automated thermocycler (Eppendorf Mastercycler Gradient; Eppendorf Poland, Warszawa, Poland). Three different targets were amplified: *nuc*, *mecA* and *blaZ*. In all three cases, the same composition of reaction mixtures was used: 2 μl of dNTPs (2.5 mM each), 2.5 μl of 10 × PCR reaction buffer (100 mM Tris-HCl, pH 8.8, 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40 and 0.5% Tween 20), 2 μl of $MgCl_2$ solution (50 mM), 1 μl of each of the 2 required primer solutions (10 μM), 1 μl of

DNA solution (prepared as described above), 0.2 μl (1 U) of polymerase *Delta* from *Pyrococcus woesei*(DNA-Gdańsk II s.c., Poland) and deionized sterile water to adjust the mixture volume to 50 μl. The primer sequences applied are presented in Table 1. In the case of all three genes, the following PCR conditions were used to generate the amplicons: 94°C for 240 sec; 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; and then 72°C for 300 sec for final extension. The amplified products were separated on 2% agarose gel in 1 × TAE buffer. The gels were stained with ethidium bromide (1 μg/m*l*), and bands were visualized in UV light.

RESULTS

The level of antibiotic resistance of the investigated group of strains was low overall (Table 2). Eighty-six out of 123 examined isolates were susceptible to all 20 tested antibiotics (70%). Cephalothin, cloxacillin, enrofloxacin, gentamicin, linezolid, rifampicin and vancomycin were active against all strains tested. The highest percentage of resistance was observed in the case of streptomycin (n=13; 10.6%) and β-lactam antibiotics: amoxicillin (n=22, 17.9%), ampicillin (n=28, 22.8%) and penicillin (n=29, 23.6%). In fact, all strains resistant to amoxicillin and ampicillin were also resistant to penicillin (n=22, 17.9%). Twenty-five of the penicillin-resistant strains were also found to carry the *blaZ* gene coding for β-lactamases, which was generally in concordance with the nitrocefin test. Three of the *blaZ*-negative strains were surprisingly positive in this test. Although the level of antibiotic resistance was low overall, the worrisome problem is isolation of several strains that could be classified as multidrug resistant (MDR) strains (Table 3) carrying resistance to 6 antibiotics and intermediate resistance to 1 agent (n=1, 0.8%), five antibiotics (n=2, 1.6%), four antibiotics and intermediate resistance to 1 agent (n=2, 1.6%) and four antibiotics (n=5, 4.0%). The strains resistant to 6 and 5 antibiotics were found to be *mecA* positive, so they could also be classified as MRSA (methicillin-resistant S. *aureus*); however, only the first one was phenotypically intermediately resistant to oxacillin in the disc diffusion assay. In the case of nonantibiotic agents tested by the microdilution method, the best results were obtained for lysostaphin (Table 4). There is no defined or officially accepted MIC value for this protein that could be used as a susceptibility breakpoint. However, some authors, for example, Kusuma and Kokai-Kun [20], classify staphylococci as resistant to lysostaphin, if the MIC value is higher than 32 μg/m*l*. The lysostaphin MIC values for all strains tested were definitely lower and were in the range from 0.008 to 0.5 μg/m*l,* so all of them can be classified as susceptible. Polymyxin B was able to inhibit the growth of most of the strains tested (n=31, 79.5%) at a concentration of 64 μg/m*l*; for 6 isolates, the MIC value for this agent was 32 μg/m*l*, and 2 strains required a higher concentration, 128 μg/m*l*, for growth inhibition.

The established susceptibility breakpoint for nisin is 32 μg/m*l* [31]. Taking into account this value, 18 out of 39 isolates from our collection were resistant to this agent's activity. However, in the case of 17 of them, the MIC value was 51.2 μg/m*l* and was not much higher than the susceptibility breakpoint value, and only in the case of 1 strain (assigned as 112), the nisin MIC value was above 51.2 μg/m*l*. The same strain was less susceptible to lysostaphin activity, which suggests a modified composition of the cell wall. However, the detailed mechanism of higher resistance to both agents was not determined and in our opinion requires further investigation. As expected, all strains tested were resistant to lysozyme activity (MIC above 2048 μg/m*l*).

DISCUSSION

The presented results indicated a considerable prevalence of antibiotic resistant strains among *S. aureus* isolated from bovine mastitis in the eastern part of Poland. Similar to studies of other authors from different regions of the world, the highest rate of resistance was detected for β-lactam antibiotics. The resistance to other tested agents was less common, which is also in agreement with the general trend observed worldwide. However, earlier published reports aimed at analysis of the antibiotic resistance of *S. aureus* strains isolated from mastitis in Poland indicated markedly higher levels of antibiotic resistance. In comparison, among the isolates examined by Malinowski *et al*. [22], the rates of resistance to penicillin, tetracycline and lincomycin were 62.3%, 41.7% and 39.4%, respectively (in each case, over 800 strains were tested). Additionally, the authors found that over 20% of the investigated strains were resistant to bacitracin and cephalexin. Higher occurrence of resistance among *S. aureus* isolates was also observed by Sachanowicz *et al.* [27], who analyzed strains from the same region, although earlier, in 2005 and 2006. High percentages of resistance against β-lactam antibiotics were also observed in several different geographical regions. Among 103 strains isolated in Turkey, the rates of resistance to penicillin, ampicillin and amoxicillin were 62.1%, 56.3% and 45.6%, respectively [35]. In the same study, about 50% resistance was found in the case of gentamicin (56.3%) and trimethoprim/sulfamethoxazole (45.6%). Additionally, eighteen of these strains were found to be phenotypically resistant to methicillin [35]. However, Aslantaş *et al.* [1], who analyzed a group of 104 strains isolated from subclinical bovine mastitis cases during 2006 to 2008 in Hatay Province, Turkey, found that 25% of them were resistant to macrolide and lincosamide (ML) antibiotics. Resistance to β-lactam antibiotics, ampicillin (59.5%) and penicillin (61.4%), was very common in a group of over 170 strains isolated in Estonia [14]. In the same study, markedly high resistance was also observed in the case of clindamycin (18.1%). Other tested antibiotics, i.e., tetracycline, erythromycin and gentamicin, were much more active with resistance rates below 10% [14]. According to results presented by Klimiene *et al*. [15], among 176 strains isolated in Lithuania, the percentages of resistance to penicillin, ampicillin and amoxicillin were 76.7%, 78.4% and 81.3%, respectively, and 38.1% of isolates were resistant to the combination of amoxicillin and clavulanic acid. The same strains were highly sensitive to cephalosporin's activity, and the rates of sensitivity rate to cephalothin and

Table 4. Bactericidal activity of the alternative agents against *S. aureus* isolates resistant to at least one of the tested antibiotics. Sensitive strains, with numbers 2 and 3, were used as a control

Code number of the strain	Lysostaphin MIC (μg/m*l*)	Nisin MIC (μg/m*l*)	Polymyxin B MIC (μg/m*l*)	Lysozyme MIC (μg/m*l*)
1	0.125	51.2	64	>2048
5	0.125	51.2	64	>2048
9	0.031	51.2	64	>2048
11	0.016	12.8	32	>2048
12	0.016	12.8	64	>2048
27	0.016	51.2	64	>2048
29	0.125	51.2	64	>2048
34	0.5	51.2	32	>2048
38	0.125	51.2	64	>2048
39	0.016	25.6	64	>2048
42	0.031	12.8	64	>2048
53	0.008	25.6	64	>2048
54	0.016	25.6	64	>2048
66	0.008	12.8	64	>2048
70	0.063	25.6	64	>2048
71	0.063	12.8	64	>2048
72	0.016	51.2	64	>2048
74	0.031	12.8	64	>2048
75	0.008	25.6	64	>2048
76	0.016	25.6	32	>2048
78	0.016	25.6	64	>2048
83	0.016	51.2	64	>2048
84	0.063	51.2	32	>2048
86	0.031	25.6	64	>2048
88	0.063	25.6	64	>2048
93	0.008	25.6	64	>2048
94	0.016	25.6	32	>2048
95	0.016	51.2	128	>2048
101	0.008	51.2	64	>2048
102	0.008	51.2	128	>2048
103	0.008	51.2	64	>2048
104	0.063	51.2	64	>2048
105	0.063	51.2	64	>2048
112	0.5	>51.2	64	>2048
113	0.008	51.2	64	>2048
116	0.031	12.8	64	>2048
119	0.016	25.6	32	>2048
2	0.063	25.6	64	>2048
3	0.008	12.8	64	>2048

cephalexin were 95.5% and 93.2%, respectively. Antibiotic susceptibility testing of 236 strains isolated from China showed an overall high level of resistance to tested antimicrobial agents [29]. Among twenty tested antibiotics, the most active ones were two aminoglycosides, kanamycin and gentamicin with sensitivity rates of only 74.6% and 69.9%, respectively. The highest rate of resistance was observed in the case of penicillin (87.3%) [29]. Recently, Gao *et al*. [10] analyzed antibiotic resistance in a group of 52 strains isolated from one Chinese herd. Interestingly, nearly all of the isolates were resistant to penicillin (96.3%) and tetracycline (98.1%), and all of them were susceptible to oxacillin, cefazolin and ciprofloxacin. A high level of resistance to penicillin (82.4%) was also observed in the case of *S. aureus* isolated from mastitis in Ethiopia; however, a very limited group, only 17 strains, was analyzed [11]. The isolates were even more resistant to clindamycin (88.2%) and were highly resistant to erythromycin (58.8%), whilst the rates of sensitivity to chloramphenicol and nalidixic acid were 58.8% and 82.4%, respectively [11]. A high prevalence of MRSA (13.1%) was observed in a group of 107 strains isolated in India [18]. The bacteria were also highly resistant to most of the other tested antibiotics, i.e., 36.4% were resistant to streptomycin, 33.6% were resistant to oxytetracycline, 29.9% each were resistant to gentamicin and ampicillin, 28.9% were resistant to penicillin and 26.2% each were resistant to chloramphenicol, pristinamycin and ciprofloxacin. The authors also revealed a high prevalence of genes coding

for different pathogenicity factors, mainly adhesins and toxins, among the tested isolates [18]. A very similar pattern of resistance was observed among 193 strains from Switzerland and 150 isolates from France [28]. The resistance to most of the 16 antibiotics tested was low overall in both countries; however, in the case of penicillin, the susceptibility rates were 77.7% and 70%, respectively. Six and 8 isolates (from Switzerland and France, 3.1% and 5.3%, respectively) were resistant to tetracycline [28]. Even lower rates of antibiotic-resistant isolates were identified in Sweden. Among a group of 109 strains, Persson *et al*. [25] tested the activities of 9 antibiotics and found only 4 (3.7%) strains resistant each to kanamycin and penicillin and only 3 strains characterized by resistance to tetracycline. No resistance was observed for other tested agents.

The observed worldwide predominance of resistance for β-lactam antibiotics is probably the consequence of the fact that they are still one of the most widely used classes of agents for treatment of bovine mastitis. Based on a questionnaire completed by 109 practicing veterinarians representing all regions of Poland, Krasucka *et al*. [16] proved that penicillins are the antimicrobials most often used in treatment of cattle infections (about 35% of cases). Additionally, in a study carried out by the same authors, a comprehensive analysis of available statistic data revealed that β-lactams are the most popular agents used in veterinary service in several other European countries including the Czech Republic, Denmark and Norway [16]. Frequent contact of bacteria with a specific antibiotic can cause an increase in resistance and decrease the effect of treatment. In fact, *S. aureus* pathogens have developed a broad spectrum of mechanisms of antibiotic resistance, which make them difficult targets even for treatment using agents from different chemical groups or combine therapy with more than 1 antibiotic from different chemical groups. The most common mechanism of β-lactam resistance is based on production of β-lactamases encoded by *blaZ*, which was confirmed in this report. The ability to produce low-affinity penicillin binding protein 2a (PBP2a) determined by the presence of the chromosomal gene *mecA* is still found incidentally among staphylococci isolated from mastitis, which is in concordance with the presented results.

The current situation in the dairy industry and veterinary service require limitation of spreading of *S. aureus* antibiotic resistance and urgent development of new antimicrobial agents that would not covered by existing mechanisms of resistance. The first goal can be achieved only if the treatment is preceded by an antimicrobial susceptibility test and selection of the most accurate agent. Such practice should be in fact commonly used in human medicine and veterinary service. Given the current state of knowledge, the most promising alternative strategies in the case of *S. aureus* diseases seem to be therapies with antimicrobial proteins and peptides [33], bacteriophages and plant- (e.g., stilbenoids and flavonoids) and animal-derived compounds (e.g., chitosan and propolis) as well as usage of vaccines and photodynamic therapy [19].

The results of our examination clearly indicate that polymyxin B, nisin and especially lysostaphin should be considered potential agents to treat infections caused by staphylococci including bovine mastitis. Polymyxin B is very effective in treatment of infections caused by G-negative bacteria. It's breakpoint MIC value for *Pseudomonas aeruginosa* is 4 μg/m*l*. The mechanism of bactericidal activity of this agent is based on destabilization of outer and inner cell membranes surrounding the cells of G-negative bacteria. G-positive bacteria are protected by a thick cell wall composed of peptidoglycan; therefore, they are more resistant to polymyxin B. Among the analyzed group of isolates, the MIC values for polymyxin B are in the range from 32 to 64 μg/m*l* (except for 2 strains with an MIC value of 128 μg/m*l*). Quite similar results were obtained by Boyen *et al*. [3], who tested the activity of polymyxin B against 24 canine MRSA isolates and observed MIC values in the range of 8–64 μg/m*l*. The observed MIC values for all strains tested in both investigations are definitely higher than the susceptibility breakpoint for this agent, established for *P. aeruginosa*, which in our opinion disqualifies using this peptide as a potential chemotherapeutic in infections caused by staphylococci, including bovine mastitis. Quite satisfactory bactericidal anti-staphylococcal activity was also found in the case of nisin (54% strains classified as susceptible). In our opinion, identification of 17 resistant strains does not disqualify this peptide as a potential agent for treatment of bovine mastitis caused by staphylococci. In fact, only in the case of one strain was the MIC value higher than 51.2 μg/m*l*, and the MIC value for other resistant strains was 51.2 μg/m*l* and close to the susceptibility breakpoint (32 μg/m*l*). Definitely, the most promising results were obtained in the case of lysostaphin. All strains tested were susceptible to its activity, and the determined MIC values were very low (from 0.008 to 0.5 μg/m*l*) and much lower the susceptibility breakpoint of 32 μg/m*l* established by Kusuma and Kokai–Kun [20]. The high anti-staphylococcal bactericidal activity of this protein has been also confirmed in many animal models of different types of infections, such as keratitis [9], endocarditis [7] and many others, that have been widely discussed in review articles by Kumar [17] and Szweda *et al.* [33]. The high potential of this protein as an alternative *S. aureus* mastitis agent has been also confirmed by other authors. Zhang *et al.* [41] revealed high *in vitro* bactericidal activity of lysostaphin against *S. aureus* isolated from mastitis in China. But, the most promising results have been published by Wall *et al.* [39], who produced transgenic cows secreting lysostaphin at concentrations ranging from 0.9 to 14 μg/m*l* of their milk. Protection against *S. aureus* mastitis appears to be achievable with as little as 3 μg/m*l* of lysostaphin in milk.

The positive therapeutic effects of using nisin in treatment of bovine mastitis have been earlier confirmed previously by several authors. Analyzing a group of 90 lactating Holstein cows with subclinical mastitis, which were randomly divided into nisin-treated (n=46) and control (n=44) groups, Wu *et al*. [40] indicated that nisin therapy had bacteriological cure rates of 90.1% for *Streptococcus agalactiae* (10 of 11), 50% for *Staphylococcus aureus* (7 of 14), 41.2% for coagulase-negative staphylococci (7 of 17) and 65.2% for all cases (30 of 46). Meanwhile, only 15.9% (7 of 44) of untreated cows spontaneously recovered. Similar results were also presented

by Cao *et al*. [5], who revealed that nisin therapy for mastitis offered a clinical cure rate similar to gentamicin. However, it has been shown that polymyxin B is well distributed throughout the bovine mammary gland and is absorbed to a significant degree into the systemic circulation from the acutely inflamed udder [42]. Less data are available about trials that have considered use of polymyxin B as a mastitis therapeutic agent. Our investigation revealed low potential for antistaphylococcal activity of this peptide. The results of the present research indicate that lysostaphin and nisin are promising alternative agents to combat staphylococcal infections. Without doubt, their potential should be investigated further.

REFERENCES

1. Aslantaş, Ö., Öztürk, F. and Ceylan, A. 2011. Prevalence and molecular mechanism of macrolide and lincosamide resistance in Staphylococci isolated from subclinical bovine mastitis in Turkey. *J. Vet. Med. Sci.* **73**: 1645–1648. [Medline] [CrossRef]
2. Bera, A., Herbert, S., Jakob, A., Vollmer, W. and Götz, F. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol. Microbiol.* **55**: 778–787. [Medline] [CrossRef]
3. Boyen, F., Verstappen, K. M. H. W., De Bock, M., Duim, B., Weese, J. S., Schwarz, S., Haesebrouck1, F. and Wagenaar, J. A. 2012. *In vitro* antimicrobial activity of miconazole and polymyxin B against canine meticillin-resistant *Staphylococcus aureus* and meticillin-resistant *Staphylococcus pseudintermedius* isolates. *Vet. Dermatol.* **23**: 381–385. [Medline] [CrossRef]
4. Brakstad, O. G., Aasbakk, K. and Maeland, J. A. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J. Clin. Microbiol.* **30**: 1654–1660. [Medline]
5. Cao, L. T., Wu, J. Q., Xie, F., Hu, S. H. and Mo, Y. 2007. Efficacy of nisin in treatment of clinical mastitis in lactating dairy cows. *J. Dairy Sci.* **90**: 3980–3985. [Medline] [CrossRef]
6. Clarke, A. J. and Dupont, C. 1992. O-acetylated peptidoglycan: its occurrence, pathobiological significance, and biosynthesis. *Can. J. Microbiol.* **38**: 85–91. [Medline] [CrossRef]
7. Climo, M. W., Patron, R. L., Goldstein, B. P. and Archer, G. L. 1998. Lysostaphin treatment of experimental methicillin-resistant *Staphylococcus aureus* aortic valve endocarditis. *Antimicrob. Agents Chemother.* **42**: 1355–1360. [Medline]
8. Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W. and Götz, F. 1999. The intracellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun.* **67**: 5427–5433. [Medline]
9. Dajcs, J. J., Hume, E. B. H., Moreau, J. M., Caballero, A. R., Cannon, B. M. and O'Callaghan, R. J. 2000. Lysostaphin treatment of methicillin-resistant *Staphylococcus aureus* keratitis in the rabbit. *Invest. Ophthalmol. Vis. Sci.* **41**: 1432–1437. [Medline]
10. Gao, J., Ferreri, M., Yu, F., Liu, X., Chen, L., Su, J. and Han, B. 2012. Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China. *Vet. J.* **192**: 550–552. [Medline] [CrossRef]
11. Haftu, R., Taddele, H., Gugsa, G. and Kalayou, S. 2012. Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia. *Trop. Anim. Health Prod.* **44**: 1765–1771. [Medline] [CrossRef]
12. Hillerton, J. E. and Berry, E. A. 2005. Treating mastitis in the cow–a tradition or an archaism. *J. Appl. Microbiol.* **98**: 1250–1255. [Medline] [CrossRef]
13. Huijps, K., Lam, T. and Hogeveen, H. 2008. Costs of mastitis: facts and perception. *J. Dairy Res.* **75**: 113–120. [Medline] [CrossRef]
14. Kalmus, P., Aasmäe, B., Kärssin, A., Orro, T. and Kask, K. 2011. Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. *Acta Vet. Scand.* **53**: 4. [Medline] [CrossRef]
15. Klimiene, I., Ruzauskas, M., Spakauskas, V., Matusevicius, A., Mockeliünas, R., Pereckiene, A., Butrimaite-Ambrozeviciene, C. and Virgailis, M. 2011. Antimicrobial resistance patterns to beta-lactams of gram-positive cocci isolated from bovine mastitis in Lithuania. *Pol. J. Vet. Sci.* **14**: 467–472. [Medline]
16. Krasucka, D., Cybulski, W., Klimowicz, A. and Dzierżawski, A. 2012. Evaluation of antimicrobial agents consumption in swine and cattle in Poland based on a questionnaire in 2010. *Med. Weter.* **68**: 106–109.
17. Kumar, J. K. 2008. Lysostaphin: an antistaphylococcal agent. *Appl. Microbiol. Biotechnol.* **80**: 555–561. [Medline] [CrossRef]
18. Kumar, R., Yadav, B. R. and Singh, R. S. 2011. Antibiotic resistance and pathogenicity factors in *Staphylococcus aureus* isolated from mastitis Sahiwal cattle. *J. Bioscience.* **36**: 175–188. [CrossRef]
19. Kurlenda, J. and Grinholc, M. 2012. Alternative therapies in *Staphylococus aureus* diseases. *Acta Biochim. Pol.* **59**: 171–184. [Medline]
20. Kusuma, C. M. and Kokai-Kun, J. F. 2005. Comparison of Four Methods for Determining Lysostaphin Susceptibility of Various Strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **49**: 3256–3263. [Medline] [CrossRef]
21. Malinowski, E. and Kłossowska, A. 2010. Mastitis caused by coagulase-negative *staphylococci* in cows. *Med. Weter.* **66**: 89–92.
22. Malinowski, E., Lassa, H., Smulski, S., Kłossowska, A. and Kaczmarowski, M. 2008. Antimicrobial susceptibility of bacteria isolated from cows with mastitis in 2006–2007. *B. Vet. I. Pulawy* **52**: 565–572.
23. Melchior, M. B., Vaarkamp, H. and Fink-Gremmels, J. 2006. Biofilms: A role in recurrent mastitis infections? *Vet. J.* **171**: 398–407. [Medline] [CrossRef]
24. Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H. and Watanabe, S. 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *J. Clin. Microbiol.* **29**: 2240–2244. [Medline]
25. Persson, Y., Nyman, A. K. and Grönlund-Andersson, U. 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *Acta Vet. Scand* **53**: 36. [Medline] [CrossRef]
26. Piepers, S., De Meulemeester, L., de Kruif, A., Opsomer, G., Barkema, H. W. and De Vliegher, S. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *J. Dairy Res.* **74**: 478–483. [Medline] [CrossRef]
27. Sachanowicz, J., Jakubczak, A. and Piechota, M. 2005. Phenotype and genotype traits of *S. aureus* strains isolated from mastitis milk samples. *Med. Weter.* **61**: 1370–1373.
28. Sakwinska, O., Morisset, D., Madec, J. Y., Waldvogel, A., Moreillon, P. and Haenni, M. 2011. Link between genotype and antimicrobial resistance in bovine mastitis-related *Staphylococcus aureus* strains, determined by comparing Swiss and French isolates from the Rhône Valley. *Appl. Environ. Microbiol.* **77**: 3428–3432. [Medline] [CrossRef]
29. Shi, D., Hao, Y., Zhang, A., Wulan, B. and Fan, X. 2010. An-

timicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis in China. *Transbound. Emerg. Dis*. **57**: 221–224. [Medline]
30. Smulski, S., Malinowski, E., Kaczmarowski, M. and Lassa, H. 2011. Occurrence, forms and etiologic agents of mastitis in Poland depending on size of farm. *Med. Weter.* **67**: 190–193.
31. Soyoğul Gürer, Ü., Sümer, B. and Rayaman, E. 2012. *In vitro* effect of nisin alone and in combination with amikacin, ceftazidime and imipenem on polymorphonuclear leukocyte functions. *Turk J. Pharm. Sci.* **9**: 171–182.
32. Szweda, P., Schielmann, M., Milewski, S., Frankowska, A. and Jakubczak, A. 2012a. Biofilm production and presence of *ica* and *bap* genes in *Staphylococcus aureus* strains isolated from cows with mastitis in the eastern Poland. *Pol. J. Microbiol.* **61**: 65–69. [Medline]
33. Szweda, P., Schielmann, M., Kotlowski, R., Gorczyca, G., Zalewska, M. and Milewski, S. 2012b. Peptidoglycan hydrolases-potential weapons against *Staphylococcus aureus*. *Appl. Microbiol. Biotechnol.* **96**: 1157–1174. [Medline] [CrossRef]
34. Tenhagen, B. A., Köster, G., Wallmann, J. and Heuwieser, W. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J. Dairy Sci.* **89**: 2542–2551. [Medline] [CrossRef]
35. Turutoglu, H., Ercelik, S. and Ozturk, D. 2006. Antibiotic resistance of *Staphylococcus aureus* and coagulase-negative staphylococci isolated from bovine mastitis. *B. Vet. I. Pulawy* **50**: 41–45.
36. Vesterholm-Nielsen, M., Olhom Larsen, M., Elmerdahl Olsen, J. and Moller Aarestrup, F. 1999. Occurrence of the *blaZ* gene in penicillin resistant *Staphylococcus aureus* isolated from bovine mastitis in Denmark. *Acta Vet. Scand.* **40**: 279–286. [Medline]
37. Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R. 2009. Mastitis detection: current trends and future perspectives. *Trends Biotechnol.* **27**: 486–493. [Medline] [CrossRef]
38. Watts, J. L. 1988. Etiological agents of bovine *mastitis*. *Vet. Microbiol.* **16**: 41–66. [Medline] [CrossRef]
39. Wall, R. J., Powell, A. M., Paape, M. J., Kerr, D. E., Bannerman, D. D., Pursel, V. G., Wells, K. D., Talbot, N. and Hawk, H. W. 2005. Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nat. Biotechnol.* **23**: 445–451. [Medline] [CrossRef]
40. Wu, J., Hu, S. and Cao, L. 2007. Therapeutic effect of nisin Z on subclinical mastitis in lactating cows. *Antimicrob. Agents Chemother.* **51**: 3131–3135. [Medline] [CrossRef]
41. Zhang, B., Shangguan, T., Ma, H., Huang, X. and Zhang, Y. 2012. Lysis of mastitis pathogens isolated from dairy cow milk samples by purified recombinant lysostaphin. *Afr. J. Biotechnol.* **11**: 4649–4659.
42. Ziv, G. and Schultze, W. D. 1982. Pharmacokinetics of polymyxin B administered via the bovine mammary gland. *J. Vet. Pharmacol. Ther.* **5**: 123–129. [Medline] [CrossRef]