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

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**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/ml. Twenty-one (54%) isolates were susceptible to nisin. The MIC value of this agent for 17 (44%) strains was 51.2 μg/ml and was not much higher than the susceptibility breakpoint value (32 μg/ml). Polymyxin B was able to inhibit the growth of the strains only at a high concentration (32–128 μg/ml). 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.

**KEYWORDS:** lysostaphin, mastitis, nisin, polymyxin B, *Staphylococcus aureus*.

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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*-acyethylmuramic 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
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.). The MIC assay for each tested strain was done 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™ 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 ml 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 μl of lysostaphin (1 U; Sigma-Aldrich Corp.) solution was added

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**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′–GCCATAGATGGTGATACGGTG | 270 | [4] |
|     |               | _nucR_: 5′–AGCCAAGCCTTGAGCAACTAAAGC |     |     |
| 2   | _mecA_        | _mecl_: 5′–AAAAATCGATGTAAGGTGG | 533 | [24] |
|     |               | _mecl2_: 5′–AGTTCTGCACTGACCGATTGTC |     |     |
| 3   | _blaZ_        | _blaZ1_: 5′–AAGAGATTGCTAGATTCTC | 517 | [36] |
|     |               | _blaZ2_: 5′–GCTTGACCACCTTTATCACGC |     |     |

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 Kokaji-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 × 10⁵ CFU/ml 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™ 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.
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 MgCl₂ solution (50 mM), 1 µl of each of the 2 required primer solutions (10 µM), 1 µl of

| 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.

| 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.
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/ml), 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 Kokei-Kun [20], classify staphylococci as resistant to lysostaphin, if the MIC value is higher than 32 µg/ml. The lysostaphin MIC values for all strains tested were definitely lower and were in the range from 0.008 to 0.5 µg/ml, 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/ml; for 6 isolates, the MIC value for this agent was 32 µg/ml, and 2 strains required a higher concentration, 128 µg/ml, for growth inhibition.

The established susceptibility breakpoint for nisin is 32 µg/ml [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/ml 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/ml. 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/ml).

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 macroline 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
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

| Code number | Lysostaphin MIC (µg/ml) | Nisin MIC (µg/ml) | Polymyxin B MIC (µg/ml) | Lysozyme MIC (µg/ml) |
|-------------|-------------------------|-------------------|-------------------------|----------------------|
| 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          | 0.016                   | 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                |
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. Its breakpoint MIC value for Pseudomonas aeruginosa is 4 µg/ml. 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/ml (except for 2 strains with an MIC value of 128 µg/ml). 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/ml. 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/ml, and the MIC value for other resistant strains was 51.2 µg/ml and close to the susceptibility breakpoint (32 µg/ml). 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/ml) and much lower the susceptibility breakpoint of 32 µg/ml 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/ml of their milk. Protection against S. aureus mastitis appears to be achievable with as little as 3 µg/ml 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.
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 DapA 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., Haesebruck1, 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., Aasbak, K. and Maedland, 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. Efficiency 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, pathological 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. Cranton, 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., Ladde, H., Gugas, G. and Kalayou, S. 2012. Prevalence, bacterial causes, and antibiofilm 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., Matusiaveicius, A., Mockeliūnaitė, R., Pereckienė, A., Butrimaitė-Ambronzevičienė, C. and Virgalius, 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. Vet. 68: 106–109.

17. Kumar, J. K. 2008. Lysostaphin: an antimicrobial agent for staphylococcal mastitis. 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. Bioscienc. 36: 175–188. [CrossRef]

19. Kurlenda, J. and Grinholec, M. 2012. Alternative therapies in Staphylococcus aureus diseases. Acta Biochim. Pol. 59: 171–184. [Medline]

20. Kusum, 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: 3265–3263. [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., Minamida, 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. 2011. Antibiotic resistance patterns to beta-lactams of gram-positive cocci isolated from bovine mastitis in Lithuania. Trop. Anim. Health Prod. 44: 550–552. [Medline] [CrossRef]

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 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. Ant-
timicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis in China. *Transbound. Emerg. Dis.* 57: 221–224. [Medline]

30. Smuski, 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. Soyogul Gurur, Ü., Sumer, 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., Koster, 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., Olholm 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 Schultz, W. D. 1982. Pharmacokinetics of polymyxin B administered via the bovine mammary gland. *J. Vet. Pharmacol. Ther.* 5: 123–129. [Medline] [CrossRef]