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Original article

Antibiotic susceptibility of methicillin-resistant and methicillin-susceptible coagulase-negative staphylococci isolated from bovine mastitis

M. Bochniarz, W. Wawron

Department and Clinic of Animal Reproduction, Faculty of Veterinary Medicine,
University of Life Sciences, Głęboka 30, 20-612 Lublin, Poland

Abstract

The aim of the present study was to evaluate the antibiotic susceptibility of methicillin-susceptible (MS) and methicillin-resistant (MR) coagulase-negative Staphylococcus (CNS) strains isolated from milk of cows with mastitis. The study was conducted on 100 CNS strains (20 MRCNS and 80 MSCNS) isolated from milk samples of 86 cows from the Lublin (Poland) region farms. Antibiotic susceptibility of microorganisms was evaluated using the disc-diffusion method on the Mueller-Hinton agar according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). The highest efficacy against MSCNS was demonstrated for cephalosporin antibiotics, i.e. cefacetril (91.3%), ceftiofur (67.5%), cefoperazone (66.3%) and cephalexin (60.0% of susceptible MSCNS strains). Moreover, a high percentage of vancomycin-susceptible strains was demonstrated (83.8%). The activity of combination of amoxicillin with clavulanic acid and gentamicin was found weaker (63.8% and 61.3% of susceptible strains, respectively). About 50.0% of MSCNS were susceptible to erythromycin, enrofloxacin and amoxicillin. A large proportion of CNS was resistant to neomycin, penicillin, tetracycline, streptomycin, lincomycin and ampicillin (28.8%, 30.0%, 31.3%, 31.3%, 33.8% and 33.8% of susceptible strains, respectively). The highest percentage of MRCNS was susceptible to vancomycin (75.0%), erythromycin (65.0%) and streptomycin (50.0%). Their susceptibility to enrofloxacin (35.0%) as well as gentamicin and tetracycline (30.0%) was markedly lower. The lowest activity was found for lincomycin and neomycin (20.0% of susceptible MRCNS strains, each).

Key words: cows, mastitis, coagulase-negative staphylococci

Introduction

Coagulase-negative staphylococci (CNS), widely spread in the natural environment, colonizing the skin and mucous membranes of animals and humans and for years considered non-pathogenic, have become the primary etiological factor of cow mastitis in many countries (Honkanen-Buzalski et al. 1994, Myllys

et al. 1998, Chaffer et al. 1999, Macovec and Ruegg 2003, Pitkälä et al. 2004, Rajala-Schultz et al. 2004, Malinowski et al. 2006, Taponen et al. 2007). Their role in inducing udder infections and inflammations in cows, sheep and goats has recently significantly increased (Deinhofer and Pernthaner 1995, Contreras et al. 2005).

The major problem of therapy of cow's mastitis caused by staphylococci is their capacity to multiply in

the host in the presence of antibiotics. This feature is determined by their exceptional adaptive ability, associated with the biofilm-forming capacity and the mechanism of acquiring drug-resistance (Łopaciuk and Dzierżanowska 2002, Markiewicz 2006).

The main causes of increasing drug-resistance include improper use of antibiotics in therapy and prevention of mastitis, particularly their use without prior identification of an etiologic factor and determination of an antibiogram. On the other hand, the emergence of new mechanisms of antibiotic resistance of microorganisms is alarming (Jabłoński 2010).

In the forties of the 20th century, defined as the "golden age" in the therapy of staphylococcal infections, all isolated strains showed full susceptibility to penicillin brought into wide clinical use (Jonsson and Wadström 1993, Łopaciuk and Dzierżanowska 2002). Within only 10 years, β -lactamase-producing staphylococcal strains emerged, which were insusceptible to penicillin. The breakthrough was observed once new, half-synthetic β -lactam antibiotics were introduced. Thanks to them, staphylococcal infections were partially controlled until methicillin- and oxacillin-resistant strains emerged (methicillin-resistant *Staphylococcus aureus* -MRSA and methicillin-resistant -MRCNS). The common feature of these staphylococci is their resistance to all classes of β -lactam antibiotics (Grzybowski and Reiss 2001, Gentilini 2002).

One of the mechanisms of β -lactam antibiotic resistance of staphylococci is the production of enzymes, which hydrolyse the β -lactam ring to form an inactive product (Bartoszewicz-Potyrała and Przondo-Mordarska 2002, Markiewicz 2006). Another extremely important mechanism of resistance to this group of antibiotics is associated with the mutation changes in genes encoding penicillin-binding proteins, which results in PBP structural changes and formation of a protein of low affinity to penicillin (PBP2a) built of 715 amino acids (Markiewicz 2006). The synthesis of PBP2a is encoded by the *mec* DNA region consisting of 2148 pairs of bases (Łopaciuk and Dzierżanowska 2002).

Moreover, methicillin-resistant staphylococci are capable of acquiring easily the genes of resistance to other groups of antibiotics. The DNA region, in which the *mec* A gene is located, contains the sequences called "gene traps" capable of binding various genes, including those of antibiotic resistance. Due to this process, methicillin-resistant staphylococci become multi-resistant strains (Łopaciuk and Dzierżanowska 2002).

The aim of the present study was to assess the susceptibility of isolated strains of methicillin-susceptible and methicillin-resistant coagulase-negative staphylococci to antibiotics most commonly used for treatment of mastitis in cows.

Materials and Methods

The study was conducted on 100 coagulase-negative staphylococcal strains: 20 methicillin-resistant (MRCNS) and 80 methicillin-susceptible (MSCNS) isolated from milk of cows with mastitis. Isolates were collected from 86 cows from the Lublin region farms – 18 herds of different housing systems (4 free-stall herds and 14 tie-stall herds) and of varying sizes (10-96 cows, 46 on average).

Prior to bacteriological examinations, clinical status of cows was evaluated, i.e. general symptoms and mammary gland changes, and milk was assessed macroscopically. Cows did not receive any drugs during the ongoing lactation. Milk was sampled according to the accepted protocol. After cleaning, washing, drying and disinfecting the teats with 70% alcohol solution, milk was collected into sterile test-tubes without preservatives, chilled to 4°C and delivered to the Department of Animal Reproduction in Lublin (Poland).

Bacteriological examination of milk was carried out according to standard procedures: culture of milk on the agar with 5% sheep blood, 24-hour incubation under oxygen conditions at 37°C; morphology of bacterial colonies and Gram-stained specimens, catalase testing (3% hydrogen peroxide, Polfa, Poland), evaluation of lisostaphin susceptibility (Sigma, USA) and free coagulase testing using rabbit plasma (Biomed, Kraków, Poland). Strains of isolated CNS were identified using the API STAPH system (Biomérieux, France).

Methicillin-resistance of all CNS strains was evaluated using the Oxacillin Resistance Screening Agar Base (Oxoid, England). Methicillin-resistant CNS strains were tested for *mecA* gene using the PCR method and for its product – PBP2, using the Penicillin-Binding Protein Latex Agglutination Test (Oxoid, England).

Antibiotic susceptibility of microorganisms was evaluated using the disc-diffusion method on the Mueller-Hinton agar according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). While choosing the antibiotics, the division into methicillin-susceptible and methicillin-resistant CNS was considered.

The MSCNS strains susceptibility to the following antibiotics was tested: amoxicillin (10 μ g), amoxicillin with clavulanic acid (30 μ g), ampicillin (10 μ g), cefacetril (30 μ g), cephalexin (30 μ g), cefoperazone (75 μ g), ceftiofur (30 μ g), enrofloxacin (5 μ g), erythromycin (15 μ g), gentamicin (10 μ g), lincomycin (15 μ g), neomycin (30 μ g), penicillin (10 u), streptomycin (10 μ g), tetracycline (30 μ g), and vancomycin (30 μ g).

According to the accepted rule of human and animal clinical microbiology, MRCNS strains were considered resistant to all β -lactam antibiotics and thus

were tested for susceptibility to the following antibiotics: enrofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), lincomycin (15 µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg).

Plates with discs were left at room temperature for 30 minutes and incubated at 35°C for 24 h. The susceptibility of a given CNS strain was measured as the zone of inhibition around the disc soaked with a particular antibiotic (Oxoid, England); based on this zone, the strains were classified as susceptible (+), medium susceptible (+/-) and resistant (-). Methicillin-susceptible *S. aureus* (29213 MSSA) and methicillin-resistant *S. aureus* (43300 MRSA) were used as reference strains.

Results

The Table 1 data demonstrate that the highest percentage of MRCNS strains was susceptible to vancomycin (75.0%), erythromycin (65.0%) and streptomycin (50.0%). The susceptibility to enrofloxacin as well as gentamicin and tetracycline was significantly lower (35.0% and 30.0%, respectively). The lowest activity was found for lincomycin and neomycin (20.0% of susceptible MRCNS, each).

Table 1. Antibiotic susceptibility of MRCNS strains.

	<i>S. sciuri</i> (8)		<i>S. xylosum</i> (6)		Other species* (6)		Total (20)	
	n	%	n	%	n	%	n	%
ENR	3	37.5	2	33.3	2	33.3	7	35.0
ER	3	37.5	5	83.3	5	83.8	13	65.0
G	3	37.5	0	0.0	3	50.0	6	30.0
L	0	0.0	2	33.3	2	33.3	4	20.0
N	0	0.0	4	66.7	0	0.0	4	20.0
S	4	50.0	4	66.7	2	33.3	10	50.0
T	4	50.0	0	0.0	2	33.3	6	30.0
Va	7	87.5	3	50.0	5	83.3	15	75.0

ENR – enrofloxacin, ER – erythromycin, G – gentamicin, L – lincomycin, N – neomycin, P – penicillin, S – streptomycin, T – tetracycline, Va – vancomycin.

* other species: *S. chromogenes* (3 strains), *S. haemolyticus* (2 strains), *S. warneri* (1 strain)

MSCNS strains were found markedly more susceptible to antibiotics compared to MRCNS. The highest efficacy was demonstrated for cephalosporin antibiotics: cefacetril (91.3%), ceftiofur (67.5%), cefoperazone (66.3%) and cephalexin (60.0% of susceptible MSCNS strains). Moreover, a high percentage was susceptible to vancomycin (83.8%). Amoxicillin was found less effective than its combination with clavulanic acid (46.3% and 63.8%, respect-

ively). About 50.0% of MSCNS strains were susceptible to erythromycin and enrofloxacin. Gentamicin was demonstrated to be slightly more active – 61.3% of susceptible strains. The remaining antibiotics showed poor efficacy to the majority of strains. The percentage of strains susceptible to streptomycin and tetracycline was found equal – 31.3%; to lincomycin – 33.8% whereas to neomycin – 28.8%. A large proportion of CNS strains was resistant to penicillin and ampicillin (only 30.0% and 33.8% of susceptible strains).

Discussion

Staphylococcus infections are difficult to treat as many strains are resistant to antibiotics used in mastitis. Due to long-term and widespread use of β -lactam antibiotics for the treatment of mastitis, many strains have become resistant to penicillin, ampicillin and amoxicillin (Aarestrup et al. 1995, Moon et al. 2007).

According to Myllys et al. (1998) and Pitkälä et al. (2004) who carried out bacteriological examinations of milk in Finland in the years 1998-2005, an increase in the number of isolated CNS strains resulted in increased numbers of strains resistant to at least one antibiotic (from 27 to 50%). Moreover, the number of

isolated CNS strains producing β -lactamase and strains with genetically-conditioned methicillin-resistance insensitive to all β -lactam antibiotics has recently increased.

In our study, significant differences in susceptibility to individual antibiotics were observed once the division into methicillin-susceptible and methicillin-resistant CNS strains was considered. MRCNS, in addition to lack of susceptibility to β -lactam anti-

Table 2. Antibiotic susceptibility of MSCNS strains.

	Number (n) and percentage of MSCNS strains susceptible to antibiotics										Total (80)	
	<i>S. haemolyticus</i> (23)		<i>S. chromogenes</i> (23)		<i>S. xyloso</i> (22)		<i>S. sciuri</i> (6)		Other species* (6)			
	n	%	N	%	n	%	n	%	n	%	n	%
AML	10	43.5	11	47.8	11	50.0	3	50.0	2	33.3	37	46.3
AMP	9	39.1	7	30.4	7	31.8	2	33.3	2	33.3	27	33.8
AMX	13	56.5	13	56.5	16	72.7	4	66.7	5	83.3	51	63.8
CEF	21	91.3	20	87.0	21	95.5	6	100.0	5	83.3	73	91.3
CFP	15	65.2	15	65.2	20	90.9	2	33.3	1	16.7	53	66.3
CL	18	78.3	18	78.3	7	31.8	1	16.7	4	66.7	48	60.0
EFT	21	91.3	16	69.6	12	54.5	3	50.0	2	33.3	54	67.5
ENR	11	47.8	16	69.6	9	40.9	2	33.3	3	50.0	41	51.3
ER	15	65.2	10	43.5	12	54.5	3	50.0	2	33.3	42	52.5
G	14	60.9	16	69.6	14	63.6	2	33.3	3	50.0	49	61.3
L	14	60.9	2	8.7	8	36.4	0	0.0	3	50.0	27	33.8
N	12	52.2	2	8.7	6	27.3	2	33.3	1	16.7	23	28.8
P	9	39.1	6	26.1	5	22.7	2	33.3	2	33.3	24	30.0
S	9	39.1	9	39.1	6	27.3	1	16.7	0	0.0	25	31.3
T	3	13.0	10	43.5	8	36.4	3	50.0	1	16.7	25	31.3
VA	19	82.6	20	87.0	17	77.3	5	83.3	6	100.0	67	83.3

AML – amoxicillin, AMP – ampicillin, AMX – amoxicillin with clavulanic acid, CEF – cefacetil, CFP – cefoperazone, CL – cephalixin, EFT – ceftiofur, ENR – enrofloxacin, ER – erythromycin, G – gentamicin, L – lincomycin, N – neomycin, P – penicillin, S – streptomycin, T – tetracycline, Va – vancomycin.

* other species: *S. warneri* (3 strains), *S. hominis* (2 strains), *S. saprophyticus* (1 strain).

biotics, showed markedly higher resistance to other chemotherapeutics, which confirms their multiple resistance. A significantly lower percentage of MRCNS was susceptible to gentamicin, enrofloxacin, lincomycin and neomycin compared to MSCNS strains. Only erythromycin and streptomycin showed higher activity against methicillin-resistant strains. Like in therapy of nosocomial staphylococcus infections in humans, vancomycin seems to be an alternative, which proved effective against the majority of MSCNS and MRCNS strains (83.8% and 75.0%, respectively). York et al (1996) have demonstrated that about 75% of strains causing nosocomial infections are resistant to methicillin. Therefore, in such cases β -lactam antibiotics are excluded and vancomycin recommended. All CNS strains (103 MR and 49 MS strains) from nosocomial infections examined by Ferreirę et al. (2003) were found susceptible to this antibiotic. Moreover, many authors observed high vancomycin susceptibility of coagulase-negative staphylococci isolated from animals (Owens and Watts 1988, Costa et al. 2000, van Duijkeren et al. 2004, Moon et al. 2007).

The evaluation of CNS susceptibility to antibiotics carried out by Moon et al. (2007) also included the division into MR and MS coagulase-negative

staphylococci (CNS). MRCNS were not resistant to more than 3 antibiotics (β -lactams excluded). On the other hand, they demonstrated markedly higher resistance to gentamicin (31.6%) and tetracycline (57.9%) compared to MSCNS (10.2% and 19.6%, respectively). Both MRCNS and MSCNS were characterized by relatively high erythromycin susceptibility (only 15.7% of MR and 8.3% of MS strains were found resistant) whereas all MRCNS were susceptible to vancomycin and ampicillin. Moreover, over a half of MSCNS strains were resistant to penicillin (59.2%) and ampicillin (57.7%), which is consistent with our findings. MSCNS strains, despite phenotypic susceptibility to methicillin, were mostly resistant to penicillin and ampicillin (only 30.0% and 33.8% of susceptible strains, respectively). The activity of cephalosporin antibiotics against the strains discussed was found to be extremely high. Cefacetil was most effective in vitro (91.3% of susceptible MSCNS strains).

The study conducted by Gentilini et al. (2002) on 123 CNS strains demonstrated that 26 isolates (21.1%) were resistant to only one antibiotic (mostly penicillin) and 9 strains showed resistance to 2 or more drugs (7.3%). All CNS studied were susceptible to gentamicin, cefalotin, and combination of ampicil-

lin and sulbactam whereas 7 isolates (5.7%) were resistant to erythromycin and 34 (27.6%) to penicillin. High resistance of CNS to penicillin and ampicillin was also reported by Owens and Watts (1988) who studies 722 staphylococcus strains isolated from milk of mastitic cows in the USA. The most commonly isolated staphylococci belonging to *S. chromogenes* i *S. epidermidis* showed low susceptibility to penicillin and ampicillin (60.3% and 80.7% of resistant strains, respectively) and high susceptibility to gentamicin, cefalotin, and combination of amoxicillin and clavulanic acid (100.0% of susceptible strains).

In study conducted by Malinowski et al. (2008), the CNS strains isolated from cows with mastitis were susceptible to: cephalexin (89.3%), cefquinom (89.2%), rifaximin (86.8%), cefacetil (86.5%) and bacitracin (86.1%). However, they were most resistant to: penicillin, ampicillin and tetracycline (29.0%, 27.6% and 18.0% of susceptible strains, respectively). The results of milk tests carried out by Pitkälä et al. (2004) in Finland showed multiple resistance (to 3 or more antibiotics) of 3.3 % of isolated CNS strains. All the strains, however, were susceptible to enrofloxacin, gentamicin, neomycin and cefalotin. Likewise, Devriese et al. (2002) did not observe the resistance of *S. chromogenes* to gentamicin, neomycin and enrofloxacin or erythromycin. Moreover, they demonstrated that *S. sciuri* resistance to lincomycin is typical of these strains, which was confirmed by our findings – all *S. sciuri* strains were insusceptible to this antibiotic.

Costa et al (2000) compared susceptibility of coagulase-negative staphylococci isolated from the mammary parenchyma of cows after slaughter with the profiles of susceptibility of CNS strains isolated from milk and demonstrated similarly high numbers of strains resistant to penicillin and ampicillin (90.0% and 84.4%, 93.2% and 95.9%, respectively); the lowest percentage of strains was resistant to vancomycin and cefalotin. In both studies, high susceptibility to gentamicin was observed (75.8% and 80.0% of susceptible strains, respectively).

The results of our study and literature data reveal highly varied susceptibility of coagulase-negative staphylococci to antibiotics and high resistance of MRCNS strains, which affects adversely the efficacy of antibiotic treatment, which is one of the essential components of mastitis control in lactating and dry cows (Rajala-Schultz et al. 2004).

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