Polish Journal of Veterinary Sciences Vol. 16, No. 4 (2013), 687–692

DOI 10.2478/pjvs-2013-0097

Original article

Resistance to methicillin of coagulase-negative staphylococci (CNS) isolated from bovine mastitis

M. Bochniarz, W. Wawron, M. Szczubiał

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

Abstract

The aim of this study was to determine the mechanisms of staphylococcal resistance to methicillin. CNS (n=100 isolates) were prepared from the mammary inflammatory secretions of 86 cows from farms located in the Lublin region.

Methicillin-resistant isolates constituted 20.0% of all CNS. *Staphylococcus sciuri* (n=8) and *Staphylococcus xylosus* (n=6) were most abundant, followed by *Staphylococcus chromogenes* (n=3), *Staphylococcus haemolyticus* (n=2) and *Staphylococcus warneri* (n=1). The *mec*A gene was found in 50.0% of MRCNS (10.0% of all CNS isolates) belonging to two species: *S. sciuri* and *S. xylosus*. All *mec*A-positive isolates contained the protein of low affinity to penicillin (penicillin-binding protein 2a – PBP2a). The enzyme hydrolysing the β-lactam ring in antibiotics was detected in 40.0% of MRCNS; 10.0% of MRCNS isolates were characterised by the presence of the *mecA* gene and ability to produce β-lactamase. The remaining 20.0% of MRCNS isolates showing phenotypic resistance to methicillin were *mec*A gene-negative and were not able to produce β-lactamase.

Key words: mastitis, cows, methicillin-resistant CNS, *mecA* gene, β-lactamase

Introduction

Coagulase-negative staphylococci (CNS), widely spread in the natural environment, colonising the skin and mucosa in humans and animals, and considered non-pathogenic for decades, has become the most important aetiological factor of bovine mastitis in many countries (Honkanen-Buzalski et al. 1994, Myllys et al. 1998, Chaffer et al. 1999, Makovec and Ruegg 2003, Pitkala et al. 2004, Rajala-Schultz et al. 2004, Malinowski et al. 2006, Taponen et al. 2007). Their role in inducing mastitis in cows, sheep and goats has recently markedly increased (Deinhofer and Pernthaner 1995, Contreras et al. 2005).

The major problem in the therapy of infections caused by CNS is their ability to form a bacterial biofilm and mechanisms of acquiring drug-resistance (Hussain et al. 1993, Łopaciuk and Dzierżanowska 2002).

One of the mechanisms of staphylococcal resistance to β-lactam antibiotics is the production of enzymes (β-lactamases) which, due to hydrolysis, break

Correspondence to: M. Bochniarz, e-mail: mariolabochniarz@interia.pl

the β-lactam ring forming the inactive product (Brakstad and Maeland 1997, Bartoszewicz-Potyrała and Przondo-Mordarska 2002). Staphylococcal resistance to β-lactam antibiotics is mainly mediated by the synthesis of the altered form of penicillin-binding protein 2 (called PBP2a or PBP2') of reduced affinity for β-lactam antibiotics. PBP2a takes over the functions of other PBPs inactivated by the antibiotic. The synthesis of PBP2a is mediated by the integration of a foreign DNA fragment of about 30 kb to the staphylococcal chromosome located between the pur and spa gene encoding the production of protein A and called the *mec* regulon. This region contains the structural gene *mecA* and regulatory genes *mecR* and *mecI*. The PBP alteration implicates the development of resistance to all β-lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams) (Hackbarth and Chambers 1989, Chambers 1997, Łopaciuk and Dzierżanowska 2002, Sawant et al. 2009).

Moreover, methicillin-resistant staphylococci are able to acquire the genes of resistance to other groups of antibiotics in a simplified way. The structure of the DNA region, where the *mecA* gene is localised, contains :gene traps; enabling the attachment of various genes, e.g. of antibiotic resistance; due to this process, methicillin-resistant staphylococci become multi-resistant (Łopaciuk and Dzierżanowska 2002).

The aim of this study was to determine the mechanisms of staphylococcal resistance to methicillin.

Materials and Methods

Isolation and identification of coagulase-negative staphylococci

The tests were performed in 100 CNS isolates prepared from mammary inflammatory secretions of 86 cows from farms of the Lublin region. The study material was collected according to the approved procedure, cooled to 4°C and delivered to the laboratory of the Department of Animal Reproduction in Lublin (Poland). The milk samples were cultured onto agar medium (BTL, Łódź, Poland) with the addition of 5% sheep blood and incubated at 37°C for 24 h aerobically. Coagulase-negative staphylococci were identified using the standard procedures, i.e. morphology of bacterial colonies, evaluation of microscopic Gram-stained preparations, catalase test, culture of the selected bacterial colonies onto Chapman agar (BTL, Łódź, Poland), assessment of lysostaphin susceptibility, and a free coagulase test using rabbit plasma (Biomed, Kraków, Poland). The CNS species were identified using the API Staph test (Biomerieux, France).

Evaluation of phenotypic methicillin resistance of coagulase-negative staphylococci

The test was conducted using Oxacillin Resistance Screening Agar Base (Oxoid, England) with the addition of ORSAB Selective Supplement. The medium solution, at a concentration of 51.75 g, was prepared in 500 ml of distilled water and sterilised in the autoclave at 121°C for 15 minutes; one vial of ORSAB Selective Supplement was added to the medium, cooled to 50°C (oxacillin concentration in the solution was 0.5 μg/ml) and poured onto Petri plates, 9-10 cm in diameter. CNS isolates were cultured on this agar. The plates were incubated at 37°C for 24-48 h. The reference *Staphylococcus aureus*, ATCC 43300, was used as a positive control.

Evaluation of genetically conditioned methicillin resistance of coagulase-negative staphylococci

DNA was isolated using the enzymatic digestion method with CTAB. The isolated DNA of individual CNS isolates resistant to methicillin was tested for the *mec*A gene using the PCR method. For amplification, primers complementary to the conservative region within the *mec*A gene were used, which restrict the 533 pz fragment. Primers for PCR were synthesized in Oligo-PAN (Warsaw, Poland). The following primer sequences for the *mec*A gene were used:
mec1: 5, AAA ATC GAT GGT AAA GGT TGG C 3,
mec2: 5, AGT TCT GCA GTA CCG GAT TTG C 3.

The reaction mixture contained : 1 U Taq Polymerase (Fermentas, Lithuania), 2.5 μl of Buffet for Taq Polymerase 10x concentrated 2.5 μl dNTPs, 1 μl of mec1 primer, 1 μl of mec 2 primer, 3 μl of MgCl2, and 9.9 μl of distilled water. The amplification products were analysed electrophoretically on 1.5% agar gel (Sigma, USA) in the presence of mass standard (100bp DNA, Fermentas, Lithuania).

Detection of the protein of low affinity to penicillin

PBP2a was detected using the Penicillin-binding Protein Latex Agglutination Test (Oxoid, England) according to the manufacturer's instructions.

Evaluation of capacity of MRCNS to produce β-lactamase

Determinations were carried out using the β-Lactamase Test (Oxoid, England). The nitrocefin-impreg-

Table 1. Mechanisms responsible for resistance of CNS to methicillin.

No of MRCNS isolates	Species	Presence of *mecA* gene	Presence of PBP2a	Production of β-lactamase
1	*S. sciuri*	+	+	–
2	*S. sciuri*	+	+	–
3	*S. sciuri*	+	+	+
4	*S. sciuri*	+	+	+
5	*S. xylosus*	–	–	–
6	*S. xylosus*	–	–	–
7	*S. chromogenes*	–	–	–
8	*S. haemolyticus*	–	–	+
9	*S. haemolyticus*	–	–	+
10	*S. warneri*	–	–	+
11	*S. chromogenes*	–	–	–
12	*S. sciuri*	+	+	–
13	*S. sciuri*	+	+	–
14	*S. xylosus*	+	+	–
15	*S. xylosus*	+	+	–
16	*S. xylosus*	–	–	+
17	*S. xylosus*	–	–	+
18	*S. chromogenes*	–	–	+
19	*S. sciuri*	+	+	–
20	*S. sciuri*	+	+	–
Total		10 isolates (50.0% of MRCNS)	10 isolates (50.0% of MRCNS)	8 isolates (40.0% of MRCNS)

nated tip of a stick was immersed in the colony of staphylococcal strains on the agar medium and a small amount of bacterial cells was collected. The results were read after 5 minutes. Stick colour change to pink-red was considered positive.

Results

One hundred isolates of coagulase-negative staphylococci from the milk of mastitic cows were tested for phenotypic resistance to methicillin. Twenty isolates (20.0% of all CNS) were qualified as the methicillin resistant staphylococci. The most abundant MRCNS were *S. sciuri* (8 isolates) and *S. xylosus* (6 isolates), followed by *S. chromogenes* (3 isolates), *S. haemolyticus* (2 isolates) and *S. warneri* (1 isolate) (Table1). The gene *mec*A was observed in 10 isolates (50.0% of MRCNS and 10.0% of all CNS isolates analysed) belonging to *S. sciuri* (8 isolates) and *S. xylosus* (2 isolates) (Fig. 1). All *mec*A-positive isolates contained the protein of low affinity to penicillin (PBP2a). The enzyme hydrolyzing the β-lactam ring in antibiotics was detected in 8 isolates of CNS (40.0% of MRCNS). The ability to produce β-lactamase was detected in *S. xylosus* (2 isolates), *S. haemolyticus* (2 isolates), *S. sciuri* (2 isolates), *S. chromogenes* (1 isolate) and *S. warneri* (1 isolates). Two *S. sciuri* isolates (10.0% of MRCNS) were characterised by the presence of the *mec*A gene and β-lactamase producing capacity. The remaining 4 isolates of MRCNS showing phenotypic resistance to methicillin were *mec*A gene-negative and were not able to produce β-lactamase: *S. xylosus* (2 isolates) and *S. chromogenes* (2 isolates).

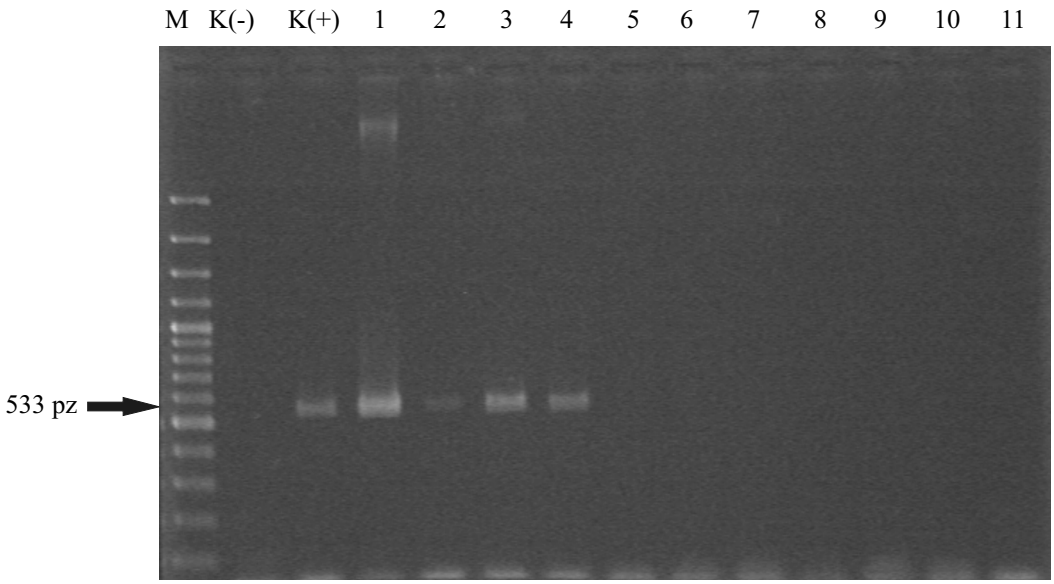

Fig. 1. Products of PCR, 533pz long, obtained on the DNA matrix of *Staphylococcus sp*. M – marker of molecular weight (100 bp DNA ladder, MBI, Fermentas Lithuania), K(-) – negative control K(+) – positive control (DNA *Staph. aureus,* methicillin-resistant ATCC 43300). Determinations of isolates 1-11 correspond to ordinal numbers in Table 1.

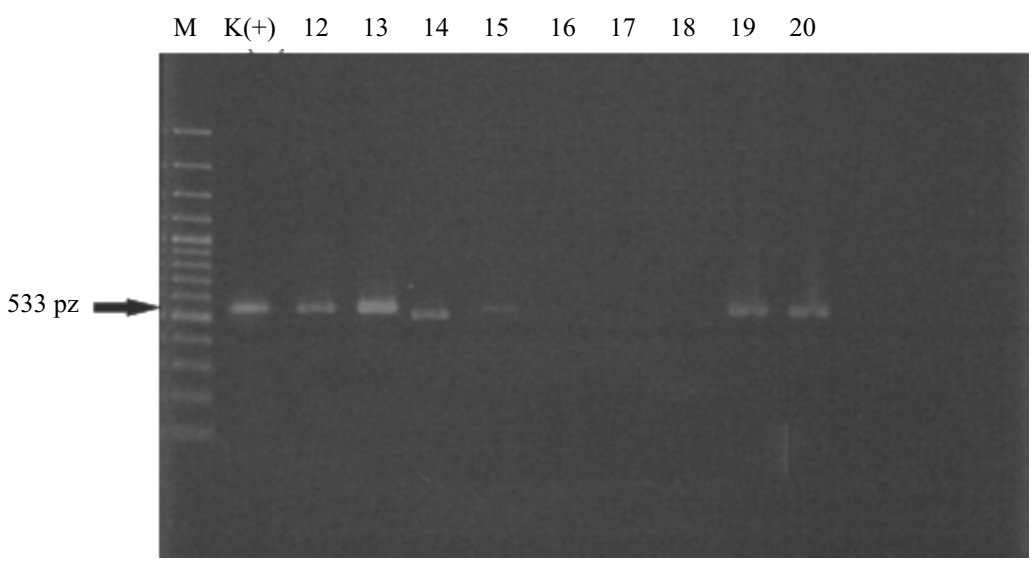

Fig. 1. continued. Products of PCR, 533 pz long, obtained on the DNA matrix of *Staphylococcus sp*. M – marker of molecular weight (100 bp DNA ladder, MBI, Fermentas Lithuania), K(-) – negative control, K(+) – positive control (DNA *Staph. aureus*, methicillin-resistant ATCC 43300). Determinations of isolates 12-20 correspond to ordinal numbers in Table 1.

Discussion

Staphylococcal infections are a relevant problem in the therapy of mastitis due to a high number of antibiotic-resistant strains (Gentilini et al. 2002, Luthje and Schwarz 2006, Bochniarz and Wawron 2011). The long-term use of β-lactam antibiotics in mastitis resulted in resistance of many isolates of CNS to penicillin, ampicillin and amoxicillin (Aaerestrup et al. 1995, Moon et al. 2007). Moreover, increased numbers of β-lactamase-producing CNS and *mec*A gene-positive CNS isolated from cow milk insusceptible to all β-lactam antibiotics, have been observed (Brakstad and Maeland 1997, Moon et al. 2007, Sawant et al. 2009, Bochniarz and Wawron 2011).

A study to determine the percentage of methicillin-resistant isolates of coagulase-negative staphylococci isolated from cows with mastitis was performed by Moon et al. (2007) in Korea. Phenotypic resistance to methicillin was demonstrated in 19 out of 736 isolates. The gene *mec*A was detected in 12 (63.2%) of 19 isolates. The remaining 7 isolates

of CNS were *mecA*-negative. According to Moon et al. (2007), isolates showing phenotypic resistance to methicillin, yet without the *mec*A gene, are β-lactamase hyper-producing isolates.

In the present study, the *mec*A gene was observed in 10 isolates, constituting 10.0% of all coagulase-negative staphylococci. Moreover, in all isolates with the *mecA* gene, the presence of its PBP2a product was detected. This finding correlates with the data presented by Louie et al. (2001); in their study, all isolates with genetically determined methicillin resistance showed agglutination indicating the presence of PBP2a (84 *mecA*-positive/84 PBP2a-positive isolates). Likewise, Miller et al. (2005) demonstrated 100% agreement between the presence of the *mecA* gene and the modified protein of low affinity to penicillin. However, the results of other studies show that the latex test for PBP2a detection can be used only for estimates, as the presence of the *mecA* gene does not always correlate with the expression of its product. Horstkotte et al. (2001) studied 201 isolates of CNS for the presence of the *mecA* gene (PCR) and PBP2a (latex test). One hundred and twenty-six isolates were found to be *mecA*-positive whereas PBP2a was detected only in 119 of these isolates; 5 isolates were poorly positive and 2 isolates were negative. On the other hand, Hussain et al. (2000) observed the opposite phenomenon. In a group of 212 *mecA*-negative CNS isolates, one isolate (*S. simulans*) showed a positive reaction in the PBP2a test. According to these findings, the only reliable method for evaluation of genetically determined methicillin resistance is thought to be the polymerase chain reaction, defined as the "gold standard" (Hussain et al. 2000, Hussain et al. 2002).

In our study, in 6 isolates, out of 10 MRCNS, without the *mec*A gene, phenotypic expression of methicillin resistance was likely to be caused by production of β-lactamase. The ability to produce this enzyme by these isolates was confirmed using a suitable test. The remaining 4 isolates of MRCNS with phenotypic methicillin resistance showed neither the presence of the *mec*A gene nor the capacity to produce β-lactamase.

The presence of *mecA*-negative CNS phenotypically resistant to methicillin is not rare. Similar examples were presented by Moon et al. (2007), mentioned above, and other authors (York et al. 1996, Gentilini et al. 2002, Van Duijkeren et al. 2004).

The study performed by Gentilini et al. (2002) on 123 isolates of coagulase-negative staphylococci isolated from cow milk revealed methicillin-resistance in 4 (3.2%) samples; however, the presence of the *mecA* gene was detected only in one CNS isolate. Devriese et al. (2002), determining the methicillin resistance among 70 isolates of *S. chromogenes* from milk of mastitic cows, did not detect the presence of the *mecA* gene in any of the isolates yet observed the ability to produce β-lactamase in 27 isolates (38.0%). Similar findings were presented by York et al. (1996) in human beings. Phenotypic methicillin resistance was found in 12 *mec*A-negative isolates identified as *S. saprophyticus*.

Furthermore, van Duijkeren et al. (2004) studied methicillin resistance in CNS isolated from various animals (cats, dogs, horses, cattle and birds) affected with different diseases. Amongst 100 isolates, 5 isolates showed phenotypic methicillin resistance, yet the *mec*A gene was found only in 4 isolates (*S. haemolyticus* – 3 isolates and *S. lentus* – 1 isolate). The causes of phenotypic methicillin resistance in *mec*A-negative isolates are unknown.

The results of our earlier study and literature data reveal highly varied susceptibility of CNS to antibiotics and high resistance of MRCNS, which adversely affects the efficacy of treatment of mastitis in cows (Moon et al. 2007, Bochniarz and Wawron 2011, Waller et al. 2011). In the field it would be necessary to evaluate antimicrobial susceptibility of each CNS isolate before therapy of mastitis caused by these microorganisms. However, MRCNS should be considered resistant to all cephalosporins, carbapenems and other β-lactam antibiotics, regardless of the in vitro test results obtained with these agents (CLSI, 2002).

References

Aarestrup FM, Wegener HC, Rosdahl VT, Jensen NE (**1995**) Staphylococcal and other bacterial species associated with intramammary infections in Danish dairy herds. Acta Vet Scand 36: 475-487.

Bartoszewicz-Potyrała M, Przondo-Mordarska A (**2002**) Cechy gronkowców koagulazoujemnych warunkujące ich chorobotwórczość. Postępy Mikrobiologii 41: 351-366.

Bochniarz M, Wawron W (**2011**) Antibiotic susceptibility of methicillin-resistant and methicillin-susceptible coagulase-negative staphylococci isolated from bovine mastitis. Pol J Vet Sci 14: 405-410.

Brakstad OG, Maeland JA (**1997**) Mechanisms of methicillin resistance in staphylococci. APMIS 105: 264-276.

Chaffer M, Leitner G, Winkler M, Glickman A, Krifucks O, Ezra E, Saran A (**1999**) Coagulase-negative staphylococci and mammary gland infections in cows. Zentralbl Veterinarmed B 46: 707-712.

Chambers HF (**1997**) Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev 10: 781-791.

Clinical and Laboratory Standards Institute (CLSI) (**2002**) Performance standards for antimicrobial disk susceptibility tests for bacteria isolated from animals. NCCLS document M32-A2, Aproved standard. 2nd ed. CLSI, Wayne, PA.

Contreras A, Sanchez A, Corrales JC, Sierra D, Gonzalo C, Paape MJ (**2005**) Mastitis in small ruminants. In:

Hogeween H (ed) Mastitis in dairy production. Wageningen Academic Publishers. The Netherlands, pp 67-74.

Deinhofer M, Pernthaner A (**1995**) *Staphylococcus spp*. as mastitis-related pathogens in goat milk. Vet Microbiol 43: 161-166.

Devriese LA, Baele M, Vaneechoutte M, Martel A, Haesebrouck F (**2002**) Identification and antimicrobial susceptibility of *Staphylococcus chromogenes* isolated from intramammary infections of dairy cows. Vet Microbiol 87: 175-182.

Gentilini E, Denamiel G, Betancor A, Rebuelto M, Rodriguez Fermepin M, De Torrest RA (**2002**) Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. J Dairy Sci 85: 1913-1917.

Hackbarth CJ, Chambers HF (**1989**) Methicillin-resistant staphylococci: genetics and mechanisms of resistance. Antimicrob Agents Chemother 33: 991-994.

Honkanen-Buzalski T, Myllys V, Pyörälä S (**1994**) Bovine clinical mastitis due to coagulase-negative staphylococci and their susceptibility to antimicrobials. Zentralbl Veterinarmed B 41: 344-350.

Horstkotte MA, Knobloch JK, Rohde H, Mack D (**2001**) Rapid detection of methicillin resistance in coagulase-negative staphylococci by a penicillin-binding protein 2a – specific latex agglutination test. J Clin Microbiol 39: 3700-3702.

Hussain M, Wilcox MH, White PJ (**1993**) The slime of coagulase-negative staphylococci: biochemistry and relation to adherence. FEMS Microbiol Rev 10: 191-207.

Hussain Z, Stoakes L, Garrow S, Longo S, Fitzgerald V, Lannigan R (**2000**) Rapid detection of mecA-positive and mecA-negative coagulase-negative staphylococci by an anti-penicillin binding protein 2a slide latex agglutination test. J Clin Microbiol 38: 2051-2054.

Hussain Z, Stoakes L, John MA, Garrow S, Fitzgerald V (**2002**) Detection of methicillin resistance in primary blood culture isolates of coagulase-negative staphylococci by PCR, slide agglutination, disk diffusion, and a commercial method. J Clin Microbiol 40: 2251-2253.

Louie L, Majury A, Goodfellow J, Louie M, Simor AE (**2001**) Evaluation of a latex agglutination test (MRSA-Screen) for detection of oxacillin resistance in coagulase-negative staphylococci. J Clin Microbiol 39: 4149-4151.

Luthje P, Schwarz S (**2006**) Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. J Antimicrob Chemother 57: 966-969.

Łopaciuk U, Dzierżanowska D (**2002**) Gronkowce metycylinooporne: mechanizmy oporności, czynniki zjadliwości oraz metody genotypowania. Postępy Mikrobiologii 41: 401-418.

Makovec JA, Ruegg PL (**2003**) Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. J Dairy Sci 86: 3466-3472.

Malinowski E, Lassa H, Kłossowska A, Smulski S, Markiewicz H, Kaczmarowski M (**2006**) Etiological agents of dairy cows' mastitis in western part of Poland. Pol J Vet Sci 9: 191-194.

Miller MB, Meyer H, Rogers E, Gilligan PH (**2005**) Comparison of conventional susceptibility testing, penicillin-binding protein 2a latex agglutination testing and mecA Real-Time PCR for detection of oxacillin resistance in *Staphylococcus aureus* and coagulase-negative *Staphylococcus.* J Clin Microbiol 43: 3450-3452.

Moon JS, Lee AR, Kang HM, Lee ES, Kim MN, Paik YH, Park YH, Joo YS, Koo HC (**2007**) Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J Dairy Sci 90: 1176-1185.

Myllys V, Asplund K, Brofeldt E, Hirvela-Koski V, Honkanen-Buzalski T, Junttila J, Kulkas L, Myllykangas O, Niskanen H, Saloniemi H, Sandholm M, Saranpaa T (**1998**) Bovine mastitis in Finland in 1988 and 1995 – changes in prevalence and antimicrobial resistance. Acta Vet Scand 39: 119-126.

Pitkälä A, Haveri M, Pyörälä S, Myllys V, Honkanen-Buzalski T (**2004**) Bovine mastitis in Finland 2001 – prevalence, distribution of bacteria, and antimicrobial resistance. J Dairy Sci 87: 2433-2441.

Pyörälä S, Taponen S (**2009**) Coagulase-negative staphylococci – emerging mastitis pathogens. Vet Microbiol 134: 3-8.

Rajala-Schultz PJ, Smith KL, Hogan JS, Love BC (**2004**) Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. Vet Microbiol 102: 33-42.

Sawant AA, Gillespie BE, Oliver SP (**2009**) Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. Vet Microbiol 134: 73-81.

Suzuki E, Hiramatsu K, Yokota T (**1992**) Survey of methicillin – resistant clinical strains of coagulase-negative staphylococci for mecA gene distribution. Antimicrob Agents Chemother 36: 429-434.

Taponen S, Koort J, Bjorkroth J, Saloniemi H, Pyörälä S (**2007**) Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism – based analysis. J Dairy Sci 90: 3301-3307.

Van Duijkeren E, Box AT, Heck ME, Wannet WJ, Fluit AC (**2004**) Methicillin-resistant staphylococci isolated from animals. Vet Microbiol 103: 91-97.

Waller KP, Aspan A, Nyman A, Persson Y, Andersson UG (**2011**) CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. Vet Microbiol 152: 112-116.

York MK, Gibbs L, Chehab F, Brooks GF (**1996**) Comparison of PCR detection of mecA with standard susceptibility testing methods to determine methicillin resistance in coagulase negative staphylococci. J Clin Microbiol 34: 249-253.