Acta Veterinaria Hungarica 52 (1), pp. 7–17 (2004)

STAPHYLOCOCCI ISOLATED FROM ANIMALS AND FOOD WITH PHENOTYPICALLY REDUCED SUSCEPTIBILITY TO β-LACTAMASE-RESISTANT β-LACTAM ANTIBIOTICS

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(Received April 26, 2003; accepted June 19, 2003)

The antibiotic resistance pattern of 1921 *Staphylococcus* strains isolated from animals and food within the last two years were examined using diffusion tests. Among them there were only 35 strains of *S. aureus* having an inhibition zone diameter of 15 mm or less, and 4 strains of coagulase-negative staphylococci (CNS) having a zone diameter of 18 mm or less to 1-μg oxacillin disk. These 39 strains were examined also by E-test to oxacillin and for the detection of the *mecA* gene by PCR in order to determine whether they might be real methicillin-resistant staphylococci. Among the 39 strains there were only two that were susceptible to penicillin by disk diffusion method; however, further examination by the penicillinase test showed that they produced β-lactamase. While 19 (15 *S. aureus,* 4 CNS) strains were resistant and 7 strains were intermediate to oxacillin in disk diffusion test, the E-test gave 8 resistant and 5 intermediate results. Six out of the 8 oxacillin-resistant strains examined by disk diffusion and E-test harboured the *mecA* gene. Thus only 6 out of the examined 1921 strains proved to be *mecA* positive. These methicillin-resistant, *mecA*-positive strains (5 of the *S. aureus* strains and 1 of the *S. epidermidis*) originated from two dairy herds. The results prove that methicillin-resistant *S. aureus* (MRSA) strains in animals are really rare in Hungary. Eighteen strains were chosen and screened for minimal inhibitory concentration (MIC) of oxacillin with or without clavulanic acid or sulbactam, and three of them produced methicillinase enzyme.

Key words: Methicillin-resistant *Staphylococcus aureus (*MRSA*),* methicillin-resistant coagulase negative staphylococci (CNS), borderline resistance

Although methicillin- (oxacillin-) resistant staphylococci often cause serious nosocomial infections and are sometimes associated with community-acquired infections in humans (Harold et al., 1998; Ma et al., 2002), they are very rare in animals. Staphylococci with this type of resistance may appear in animals because of

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the use of β-lactamase-resistant β-lactam antibiotics (e.g. cloxacillin) in dry cow therapy, and because of the ability of staphylococci to acquire resistance genes located within plasmids, transposons or other mobile DNA elements.

Oxacillin resistance in staphylococci is usually mediated by an acquired gene (*mecA*) (Brakstad and Maeland, 1997). This gene is present in the chromosome of methicillin-resistant staphylococci and encodes a penicillin-binding protein (PBP2' or PBP2a) possessing decreased affinity for binding to all antibiotics of β-lactam type. The *mecA* gene is carried by a mobile genetic element designated staphylococcal cassette chromosome *mec* (SCC*mec*), of which four forms are distinguished according to their size and genetic composition (Enright et al., 2002). SCC*mec* is composed of the *mec* complex encoding methicillin resistance and the recombinase gene complex designated *ccrA* and *ccrB* responsible for its integration and excision (Katayama et al., 2000; Hiramatsu et al., 2001; Ma et al., 2002). The *mecA* complex is formed by the *mecA* gene, by a copy of insertion sequence (IS*431mec*) and by regulator genes, *mecI* that strongly represses the transcription of *mecA* gene and *mecR1* that encodes a transmembrane inducer of *mecA* consisting of membrane-spanning and penicillin-binding domains. The *mecR1* and *mecI* are very similar to the *blaR1* and *blaI*, which regulate β-lactamase production and are able to regulate the expression of the *mecA* gene too (Hackbarth and Chambers, 1993). In some strains the set of regulatory genes is partially deleted, and besides the *mecA* only a part of *mecR1* gene is left. Depending on the form of *mecR1* and *mecI* genes five different classes of *mec* complex have been described (Lim et al., 2002). The presence of an intact *mecI* gene causes repression of *mecA* transcription, and because of the low level of PBP2' production strains may show susceptibility or a typical heterogeneous low-level oxacillin resistance (Dickinson and Archer, 2000; Katayama et al., 2001). Besides the regulator genes (*mecR1, mecI* and *blaRI, blaI* systems) the expression of methicillin resistance may be influenced by *fem* (factors enhancing methicillin resistance) or *aux* (auxiliary) factors. Partial deletion or inactivation of one or more regulator genes or additional factors may cause changes in the phenotype of staphylococci harbouring *mecA* gene, and it is believed that such isolates may appear as heterogeneously resistant in their expression of resistance to β-lactam agents. While a few clinical isolates of methicillin-resistant staphylococci express homogeneous (high-level) oxacillin resistance, the majority of the isolates are heteroresistant. Homoresistance means that more than 1% of the cell population grow on tryptic soy agar supplemented with 50 μg/ml of methicillin at 37 °C for 72–96 h. Heteroresistance means that less than 1% of the bacteria grow in the above-mentioned circumstances (Hartman and Tomasz, 1986). Test conditions have an important role in the detection of heteroresistant strains. Phenotypic methicillin-resistant staphylococci that do not have *mecA* gene may overproduce β-lactamase, or may produce a novel oxacillinase. These types of resistance usually appear as low-level or borderline resistance

(BORSA = borderline resistant *Staphylococcus aureus*) like the resistance of heteroresistant *mecA* positive strains.

Since January 2001 we have examined staphylococcal strains with phenotypically decreased susceptibility to oxacillin using oxacillin E-test (Epsilometer test) strips to determine minimal inhibition concentration (MIC), and for the detection of the *mecA* gene by polymerase chain reaction (PCR) technique. The oxacillin-hydrolysing activity of some strains was also measured.

Materials and methods

Origin of staphylococcal strains. A total of 1005 staphylococcal strains were isolated from carcasses or samples of different animal species (867 strains from bovine mastitis, 67 from poultry, 18 from pigs, 13 from horses and 40 from dogs). Another set of staphylococcal strains (915) was isolated from foods examined at the National Food Investigation Institute, and strains having phenotypically decreased susceptibility to methicillin were further investigated in the Central Veterinary Institute.

Strains were cultured, identified, tested for antibiotic susceptibility and for the presence of *mecA* as was previously described (Jaffe et al. 2000; J. Kaszanyitzky et al., 2003).

The isolates were classified as resistant when the inhibition zone to 10 μg penicillin disk was less than 29 mm. All of the 35 *S. aureus* with ≤ 15 mm and 4 coagulase-negative *Staphylococcus* (CNS) strains with ≤ 18 mm zone sizes to 1 μg oxacillin disk were drawn into this study (Tables 1 and 2). This selection differs from the generally accepted interpretation where strains of *S. aureus* with a zone diameter of ≥ 13 mm and CNS with a zone diameter of ≥ 18 mm are interpreted as susceptible and values of resistance are ≤ 10 mm and ≤ 18 mm to 1 μg oxacillin disk by the National Committee for Clinical Laboratory Standards (NCCLS, 2001, 2002). *S. aureus* strains for which the MIC is ≤ 2 μg/ml are considered susceptible and those for which the MIC is ≥ 4 μg/ml are considered resistant. These values for CNS isolates are ≤ 0.25 μg/ml and ≥ 0.5 μg/ml, respectively.

Beta-lactamase test. This was performed with Beta-lactamase Diagnostic Tablet (Rosco, Taastrup, Denmark) as recommended by Rosco.

Reference strains. The methicillin-resistant *S. aureus* reference strain ATCC 33591 from Oxoid was used as positive control in PCR. The reference borderline methicillin-resistant strain was *S. aureus* VU 94, kindly provided by D. S. Kernoble (Vanderbilt University, Nashville, USA).

Antimicrobial agents. Methicillin (Bristol Laboratories, Paris), oxacillin (Sigma Chemical Company, St. Louis, USA), clavulanic acid (Pfizer), sulbactam (SmithKline Beecham) were used. The chromogenic cephalosporin, nitrocefin was purchased from Oxoid Ltd. (London).

Macrodilution method. MIC values were determined by a macrodilution method using suspension of *S. aureus* strains at a concentration of 10^5 CFU/ml in Mueller-Hinton broth supplemented with 2% NaCl. Antibiotics were added in serial twofold dilutions ranging from 128 to 0.125 µg/ml. Bacterial growth was tested after 24-h incubation at 35 °C. Alternatively clavulanic acid and sulbactam were added at a concentration of 5 and 20 µg/ml, respectively.

Identification of β-lactamase production. Bacteria were grown in 1% of CY (Casein Yeast) broth buffered with phosphate buffer (pH 7.4), with or without 0.5 µg/ml methicillin as the inducer of β-lactamase production. Cells were harvested by centrifugation and membrane fraction was isolated.

Isolation of bacterial membranes. Bacterial membrane fractions were prepared according to Barabás et al. (1988).

Detection of β-lactamases with SDS-PAGE. SDS-polyacrylamide gel electrophoresis was performed according to the method described by Laemmli (1970). Separation was performed in 13% gel in a continuous SDS-Tris-glycine buffering system. MultiMark® Multi-Coloured Standard (Invitrogen) was used to estimate the molecular weights of the proteins. After electrophoresis, the gels were soaked in renaturating buffer containing 0.1 mM $ZnSO_4 \cdot 7H_2O$ and 1% Triton-X-100 in 0.01 M phosphate-buffer (Massida et al., 1991). Six to 8 h of incubation at 37 °C was necessary to renature staphylococcal β-lactamases. The gel was placed on 1% agar plate containing 100 µg/ml nitrocefin: enzymatic activity was detected directly in gels using colour change of nitrocefin.

Measurement of oxacillin-hydrolysing activity: 50 µl of isolated membranes was mixed 80 µg/ml oxacillin in 50 µl. After 30 min of incubation at 37 °C, the decrease of oxacillin concentration was measured by the agar diffusion method using *Bacillus subtilis* ATCC 6633 strain as test organism. The oxacillin-hydrolysing activity was determined after 24 h of incubation at 37 °C.

Results

In 2001 and 2002 the antibiotic resistance patterns of 1921 staphylococcal strains were examined using the disk diffusion test. The strains of *S. aureus* having an inhibition zone of 15 mm or less around the oxacillin disk and CNS strains with an inhibition zone diameter of 18 mm or less were tested for MIC with E-test and for the presence of *mecA* gene with PCR. In the last two years we found *S. aureus* strains in 35 cases (21 from animals, 14 from food), *S. epidermidis* in 2 cases (18631, 30304 from animals), *S. warneri* in one case (1458/141 from food) and *S. sciuri* in one case (28518/189 from food) that were assumed to have *mecA* gene. All strains but two were resistant to penicillin by the disk diffusion test but all of them produced β-lactamase enzyme (Tables 1 and 2).

Table 1

Data of strains isolated from animals with ≤ 15 mm inhibition zone diameters to oxacillin

Strains	Origin	Antimicrobial susceptibility test					Oxacillinase	*mecA* gene	Resistance to other drugs
		Disk diffusion test (d/mm)			E-test (μg/ml)				
		penicillin	oxacillin						
13218/216	cow	12	11	I	1.5	S	–	–	–
13218/255	cow	13	10	R	1.5	S	–	–	–
15884/156	cow	28	9	R	0.5	S	–	–	R: SXT
15884/197°	cow	30	15	S	0.5	S	–	–	R: SXT
15884/206	cow	23	13	S	0.38	S	–	–	–
17052/11	cow	11	10	R	3.0	I	–	–	–
17052/81	cow	14	14	S	1.0	S	–	–	–
17258/11°	cow	30	13	S	1.5	S			
17766/78	cow	15	12	I	1.0	S	–	–	–
17766/87	cow	15	11	I	1.5	S	+	–	R: Li
18631*	cow	12	12	R	1.0	S		–	–
19599/3A	cow	11	9	R	1.5	S	+	–	–
19599/3B	cow	11	9	R	1.5	S	+	–	–
26061	poultry	27	12	I	1.0	S		–	R: Te
29001	horse	26	9	R	1.5	S		–	
13535	cow	6	6	R	64	R		+	R: Cl, XNL I: Te, Ery
16480/4	cow	10	6	R	48	R		+	R: Cl, XNL, Ery, Te, I: Neo
16480/5	cow	12	6	R	48	R		+	R: Cl, Ery, Te, I: Neo
27453/9	cow	25	6	R	3	I		–	–
27453/17	cow	27	6	R	3	I		–	–
24069/2	cow	25	10	R	16	R		+	R: Ery, Te, I: Neo
30195/4	cow	11	6	R	16	R		+	R: Ery, Te
30304*	cow	14	6	R	3	R		+	R: XNL, Cl, Ery, Te

*CNS (coagulase-negative staphylococci); °Strains that produce β-lactamase but are susceptible to penicillin in disk diffusion test. Abbreviations: S, susceptible; R, resistant; I, intermediate; XNL, ceftiofur; Cl, cephalexin; Ery, erythromycin; Li, lincomycin; Neo, neomycin; SXT, sulphonamide/trimethoprim; Te, tetracycline

Four strains from animals and 9 strains from food proved to be susceptible to oxacillin both in disk diffusion and E-test. Ten strains from animals and 3 from food were intermediately susceptible or resistant to oxacillin in disk diffusion test but susceptible in E-test. Among them three *S. aureus* strains produced oxacillinase enzyme, too. Out of the 39 strains, 3 from animals and 2 from food showed either intermediate susceptibility or resistance to oxacillin in disk diffusion test and intermediate susceptibility in E-test.

Table 2

Data of strains isolated from food with ≤ 15 mm inhibition zone diameters to oxacillin

Strains	Origin	Antimicrobial susceptibility test					Oxacillinase	*mecA* gene	Resistance to other drugs
		Disk diffusion test (d/mm)			E-test (μg/ml)				
		penicillin	oxacillin						
1458/133	sausage	18	14	S	0.25	S		–	
1458/141*	sausage	19	13	R	0.25	S		–	
2461/1457	sausage	9	12	I	0.5	S	–	–	
2986/416	sausage	0	14	S	0.5	S	–	–	
10517/1223	chicken meat	13	13	S	0.38	S		–	R: Te
14452/2122	sausage	13	15	S	0.25	S		–	
14452/2199	sausage	13	15	S	0.25	S	–	–	
16391/4091	fresh milk	16	13	S	0.38	S	–	–	
16391/4094	fresh milk	23	12	I	0.5	S	–	–	
16391/4241	fresh milk	25	13	S	0.25	S	–	–	
16391/3811	sausage	15	15	S	0.25	S	–	–	
17749/2724	sausage	13	14	S	0.25	S		–	
28518/189*	sausage	26	14	R	1.0	R		–	
18182/2287	sausage	13	12	I	3	I		–	R: Cl
18182/2394	sausage	13	10	R	3	I		–	I: Cl
31755/135	fresh milk	25	9	R	4	R		–	

*CNS

Eight strains were resistant both in disk diffusion and in E-test. Two of them from food were *mecA*-negative and 6 strains from animals harboured *mecA* gene. The MIC proved to be 3 μg/ml in the *mecA*-positive *S. epidermidis* strain and 16 to 64 μg/ml in the five *S. aureus* strains. Thus only 6 of the examined 39 strains proved to be *mecA*-positive: 5 of them were *S. aureus* from the same dairy herd and one was *S. epidermidis* from another herd.

For the investigation of penicillinase hyperproducer phenotypic methicillin resistance, 18 strains were randomly selected and screened for MIC of oxacillin with or without clavulanic acid or sulbactam. According to the measured values we estimated that the 19599/3B strain is borderline methicillin resistant, because the MIC value of oxacillin was reduced from 1 μg/ml to 0.25 μg/ml in the presence of clavulanic acid or sulbactam (Table 3). The 19599/3A strain also proved to be borderline methicillin resistant in the case when a medium containing 2% NaCl was used. Surprisingly, in the case of the 17766/87 strain the MIC of oxacillin was 2 μg/ml when the medium was supplemented with 2% NaCl, and it did not decrease with clavulanic acid or sulbactam. The remaining 15 strains were susceptible to oxacillin.

Table 3

MIC values of the strains examined

Strains	MIC (MH)			MIC (MH + 2% NaCl)		
	OXA	OXA + S	OXA + C	OXA	OXA + S	OXA + C
17766/87	0.5	0.5	< 0.25	2	2	2
19599/3A	1	0.5	0.5	1	0.25	0.25
19599/3B	1	< 0.25	< 0.25	1	< 0.25	< 0.25

C = clavulanic acid; S = sulbactam

Using SDS-PAGE it was found that in the bacterial supernatant a larger (33 kDa) protein band and in the membrane fractions two protein bands appeared, one with a molecular mass corresponding to the molecular mass of the β-lactamase in the supernatant and one with a molecular mass of 12 kDa. The β-lactamases were present both in the induced and non-induced samples. The 17766/87 strain does not produce extracellular β-lactamase and in the membrane fraction only the β-lactamase with lower molecular mass was present.

Performing bioassay with our samples (Table 4) both the membrane fractions and supernatants of 19599/3A and 19599/3B strains showed slow oxacillin-hydrolysing activity, and this could be increased by the addition of methicillin as inductor. The molecular mass of the enzyme in the supernatant was the same as that of the larger β-lactamase in the membrane. Otherwise both enzymes can split oxacillin. Therefore we suppose that these enzymes are the secreted and membrane-bound form of the same enzyme, which could be functionally equal with methicillinase.

Table 4

Oxacillin hydrolysis of the examined strains (hydrolysed oxacillin μg/ml/10 μg protein/hour)

Sample	17766/87	19599/3A	19599/3B
Supernatant	0	0.553	0.4
Induced supernatant	0	0.976	0.89
Membrane	0.635	2.7	1.36
Induced membrane	2.67	11.04	11.02

Discussion

Although methicillin-resistant *S. aureus* (MRSA) appears to be primarily a human pathogen and is infrequent in animals, there are some veterinary reports on mastitis in cows and lesions in horses and dogs caused by MRSA and about their colonisation in dogs and cats (Scott et al., 1988; Cefai et al., 1994; Devri-

ese, 1975; Devriese et al., 1997; Hartmann et al., 1997; Gortel et al., 1999; Seguin et al., 1999; Tomlin et al., 1999; Yasuda et al., 2002). In most cases the human origin of the infection could be demonstrated.

The most important risk factor for the emergence of resistance in bacteria is the contact with antibiotics. Therefore, the use of cloxacillin in dry cow treatment and mastitis therapy may contribute to the selection and spread of methicillin resistance in staphylococci of animal biotypes, too.

The present study was performed to monitor the development of methicillin resistance in staphylococci from animals and food. According to the guidelines of the NCCLS (2001, 2002), in the antibiotic susceptibility test we applied stricter criteria for *S. aureus* strains, e.g. we chose 15 mm inhibition zone instead of 12 mm. To prove that MRSA strains in animals are also rare in Hungary, all of the *S. aureus* isolates with ≤ 15 mm zone diameter and CNS with ≤ 18 mm zone diameter were subjected to further examinations.

We thought this reasonable because serial examinations had proven that not only several genes but also the test conditions had major effects on the expression of methicillin resistance in staphylococci, as most strains were heteroresistant. Repression of *mecA* transcription by *mecI* may be so strong among heteroresistant strains that only a little or no production of PBP2a enzyme can be detected. On the other hand, it is also possible that test conditions are not 'favourable' enough to express higher-level resistance (Brown, 2001). Methicillin resistance may appear as low-level resistance in those staphylococci that do not contain *mecA* gene. The so-called borderline-resistant strains that produce large amounts of β-lactamase and may hydrolyse methicillin, oxacillin and sometimes β-lactamase inhibitors as well appear as low-level methicillin resistant strains (McDougal and Thornsberry, 1986). However, it was shown that besides β-lactamase hyperproduction, a small increase in intrinsic resistance is required for the emergence of borderline resistance (Barg et al., 1991; Gál et al., 2001). Among clinical BORSA isolates methicillinase-producing strains were detected (Massida et al., 1991).

In order to differentiate the real methicillin-resistant staphylococcal strains from those strains showing only phenotypic methicillin resistance, strains presumably having *mecA* were examined for MIC with E-test and for *mecA* gene with PCR. It is very important to recognise the real oxacillin-resistant staphylococci because β-lactams may appear active against oxacillin-resistant staphylococci *in vitro*, but they are not effective in clinical practice. The reason why β-lactams are likely to fail is that a resistant subpopulation is selected out from the heteroresistant phenotype of *mecA*-positive staphylococci and grows during the treatment (Chambers et al., 1984).

Among the 39 strains there were only 2 that were susceptible to penicillin by disk diffusion method (15884/197, 17258/11) but the result of the penicillinase-test showed that they produced β-lactamase. *S. aureus* strains can produce

four types of β-lactamase (A, B, C and D). Although the four types of enzymes exhibit differences in activity of hydrolysing various penicillin and cephalosporin substrates (Zygmunt et al., 1992), in most cases disc diffusion antibiotic resistance tests using 10-μg penicillin disk are able to detect β-lactamase producing strains. Two out of the 18 tested strains (19599/3A and 19599/3B) were found to be β-lactamase hyperproducer. These strains produced methicillinase. This enzyme is capable of hydrolysing penicillinase-resistant penicillins at a slow rate, causing borderline methicillin resistance. The third strain, 17766/87, was found to be borderline resistant, too. This strain did not produce methicillinase, and the oxacillin hydrolysis of its membrane fraction was weaker even at induction than that of the other strains. According to these findings, we can state that the borderline methicillin resistance of this latter strain is due to an intrinsic mechanism.

The E-test was more sensitive and specific for detecting *mecA*-positive staphylococci than the disk diffusion test, but PCR was the most reliable and specific.

As PCR is regarded as the 'gold standard' for the detection of *mecA* gene, all of the 39 strains were examined by this method. Several genetic determinants are necessary for maximal expression of methicillin resistance but only the *mecA* gene can be found in all methicillin-resistant strains, and it is absent from methicillin-susceptible staphylococci.

All examined *S. aureus* strains with a MIC between 0.25–3 μg/ml were *mecA* negative by PCR. One of the *S. epidermidis* strains with 3 μg/ml MIC had *mecA* gene. Two strains, which were resistant to methicillin both in disk diffusion and E-test, were *mecA* negative.

The six methicillin resistant, *mecA*-positive strains showed resistance not only to all β-lactams but also to erythromycin and tetracycline, and two of them were intermediately resistant to neomycin. Methicillin-resistant staphylococci become multiresistant rather frequently by the activity of IS*431mec* insertion sequence that serves as a chromosomal deposit site for multiple resistance genes. In this study, the *mecA*-positive strains came from two herds.

It is important that the 6 strains were only from two herds, and not from 6 different places.

These findings are in agreement with previously published reports dealing with the presence of methicillin-resistant staphylococci in animals and in products of animal origin. Application of β-lactamase resistant β-lactam antibiotics should need prudent consideration for preventing the spread of staphylococci bearing *mecA* gene in animals.

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