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Resistance to Methicillin in Coagulase-negative Staphylococci and Its Detection

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Abstract

Resistance of staphylococci to methicillin is important especially in the case of *Staphylococcus aureus* isolates. Its impact in veterinary medicine is not exactly specified in coagulase-negative staphylococci; however, these staphylococci may represent an important reservoir of resistance genes.

The study aimed at detecting resistance to methicillin in coagulase-negative staphylococci from raw materials and foodstuffs of animal origin and assessing the tests frequently used to determine this resistance**.**

Coagulase-negative staphylococci (198 isolates of 12 species) were tested. Resistance to methicillin was determined by the disk diffusion method using oxacillin and cefoxitin disks, microdilution method, detection of PBP2a and the *mecA* gene. Of the tested isolates, 109 (55.1%) were classified as resistant by the diffusion test with oxacillin, 32 isolates (16.2%) by the test with cefoxitin and 50 isolates (25.3%) on the basis of oxacillin minimum inhibitory concentration (MIC). No resistant isolates were incorrectly identified as susceptible when using the disk diffusion method with oxacillin (sensitivity of 100%). However, apart from 22 correctly classified resistant isolates, another 87 isolates were incorrectly identified as resistant as well (specificity of 50.6%). The test with cefoxitin showed the lowest (45.5%) sensitivity in determination of resistant isolates. By contrast, this test was the most precise in classification of resistant isolates (specificity of 87.5%). When using the microdilution method, resistant strains were identified with the sensitivity and specificity of 68.2% and 80.1%, respectively.

The results revealed substantial variability of methicillin-resistant isolates ranging from 16.2% to 55.1%, depending on the phenotyping methods and recommended interpretation criteria used. Therefore, it is advisable to reconsider the current interpretation criteria in the case of coagulase-negative staphylococci of animal origin (with the exception of *S. epidermidis*).

Coagulase-negative staphylococci, animals, resistance, methicillin

One of the current problems in both human and veterinary medicine is bacterial resistance to antimicrobial agents. The failure to treat infectious diseases caused by resistant bacteria leads to increased morbidity and mortality and also poses a serious epidemiological risk. Moreover, the economic impact is not negligible. From the point of view of clinical importance of antibiotic resistance, the most important bacteria are, among others, methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci.

The importance of methicillin-resistant coagulase-negative staphylococci in veterinary medicine has not been defined. However, these strains may represent a reservoir of resistance genes (Vengust et al. 2006; Busscher et al. 2006), which is why they are so important. Coagulase-negative staphylococci are considered to be major pathogens causing mastitis, resulting in farmers' economic losses. They may also be isolated from infections in other animal species, such as poultry, dogs, cats and pigs (Aarestrup and Schwarz 2006).

Resistance to oxacillin/methicillin is determined by the presence of the *mecA* gene encoding penicillin-binding protein 2a (PBP2a) with very low affinity to beta-lactam

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antibiotics (Chambers 1997). Detection of the *mecA* gene by PCR or PBP2a by latex-agglutination test may explicitly identify methicillin-resistant strains (Hájek et al. 2002; van Leeuwen et al. 1999). Identification is problematic if the disk diffusion or microdilution methods are used and isolates are classified according to interpretation criteria. For coagulase-negative staphylococci, with the exception of *S. lugdunensis*, different interpretation criteria are stated by the Clinical and Laboratory Standards Institute document (CLSI 2007) than for *S. aureus* (MIC 0.25 mg/l and 2 mg/l, respectively). Moreover, such resistance is based predominantly on examination of human isolates. Therefore, the objective of this study was to determine the resistance to methicillin in coagulase-negative staphylococci from raw materials and foodstuffs of animal origin and to assess the accuracy of five tests frequently used to determine resistance to methicillin: 1) *mec*A gene detection as a reference, 2) and 3) oxacillin and cefoxitin disk tests, respectively (inhibition zone sizes), 4) microdilution test (MIC to oxacillin) and 5) PBP2a detection for non-human coagulase-negative staphylococci isolates. Sensitivity, specificity and interpretation criteria of the above-mentioned methods for 12 species of coagulase-negative staphylococci were evaluated.

Materials and Methods

Staphylococcal isolates

Coagulase-negative staphylococci (198 isolates) from raw materials and foodstuffs of animal origin were collected in 2003–2006. The samples were taken from milk (both fresh and pasteurized), dairy products (cheese, butter) and swabs from dairy technology.

The scheme of identification established in the laboratory: Analytical samples from raw materials and foodstuffs of animal origin were cultured in parallel on Baird-Parker agar (Merck, Darmstadt, Germany) and KRANEP agar (Merck). A maximum of five suspect morphologically dissimilar colonies of *Staphylococcus* spp. were cultured on blood agar plates containing 5% sheep blood and identified using the catalase test, oxi test, bacitracin and furazolidone susceptibility disk diffusion tests and coagulase test, and, biochemically by the STAPHYtest 24 identification system (Pliva-Lachema, Brno, Czech Republic). Isolates of the same species from a single sample were not undertaken in this study. The isolates, belonging to 12 species of staphylococci, were stored in tryptone soya broth (TSB; Oxoid, Basingstoke, UK) supplemented with 20% glycerol at -80 °C until studied.

Tests for the determination of resistance to methicillin

As recommended by Barry and Thornsberry (1991), for the identification of methicillin-resistant *S. aureus* strains, oxacillin should be preferred to cloxacillin, methicillin and other penicillinase-resistant antibiotics. Therefore, oxacillin, not methicillin, was used in the compared tests. The MIC values for staphylococci to oxacillin were determined by the standard microdilution method (CLSI 2007) with oxacillin concentrations ranging from 0.03 to 4 mg/l. The MIC susceptibility/resistance interpretation criterion was defined as a concentration of 0.25 mg/l, based on the CLSI guidelines (CLSI 2007). Reference strains *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 served for protocol quality control. Furthermore, two disk diffusion methods were used, with 1 µg oxacillin (Oxoid) and 30 µg cefoxitin (Oxoid) disks using Mueller-Hinton agar (Trios, Prague, Czech Republic). Resistant isolates were identified based on inhibition zone sizes of ≤ 17 mm for oxacillin and ≤ 24 mm for cefoxitin, respectively (CLSI 2007). Reference strain *Staphylococcus aureus* ATCC 25923 was used for quality control of the disk methods. Detection of PBP2a by latex agglutination (Cavassini et al. 1999; Felten et al. 2002) was performed with the MRSA-Screen test (Denka Seiken Co., Japan). The PBP2a detection was confirmed by the *mecA* gene detection by PCR (primers *mecA*-F:5´-TCCAGATTACAACTTCACCAGG-3´ and *mecA*-R:5´-CCACTTCATATCTTGTAACG-3´; Oliveira and De Lencastre 2002) using a modified approach (Sauer et al. 2008). Template DNA was obtained by lysis of cells from bacterial culture induced by boiling. Briefly, isolates were grown on blood agar (Trios) for 24 h, one single colony was picked up, resuspended in 100 µl of sterile deionised water and heated at 99 °C for 15 min under mild shaking in the Thermomixer comfort (Eppendorf). Then the tubes were centrifuged (1006 *g*, 5 min) to sediment ballast material whereas the supernatant containing crude extract of bacterial DNA was used.

When assessing the methods, the *mecA* gene detection was considered the gold standard for identification of methicillin-resistant isolates.

Evaluation of tests

The tests were compared on the basis of correct and incorrect identification of resistant and susceptible isolates. The following indicators were used: 1) specificity – the ability of a test to detect truly susceptible isolates, and 2) sensitivity – the ability of a test to detect truly resistant isolates. Identification of false-susceptible isolates with respect to the gold standard was considered as a major error and of false-resistant isolates as a minor error.

Table 1. Assessing the resistance of *Staphylococcus* spp. to oxacillin using oxacillin and cefoxitin disks and oxacillin MIC

Species	No. of strains	Oxacillin zone		Oxacillin disk	Cefoxitin zone		Cefoxitin disk	Oxacillin MIC		Oxacillin MIC
		≤ 17 mm	≥ 18 mm	Resistance (%)	≤ 24 mm	≥ 25 mm	Resistance (%)	≥ 0.5 mg/l	≤ 0.25 mg/l	Resistance (%)
S. auricularis	13	10	3	76.9	1	12	7.7	1	12	7.7
S. saprophyticus subsp. *bovis*	17	15	2	88.2	2	15	11.8	12	5	70.6
S. epidermidis	20	16	4	80	11	9	55	16	4	80
S. haemolyticus	19	5	14	26.3	9	10	47.4	0	19	0
S. hominis subsp. *hominis*	10	2	8	20	0	10	0	0	10	0
S. hyicus	17	0	17	0	0	17	0	0	17	0
S. chromogenes	21	12	9	57.1	1	20	4.8	1	20	4.8
S. saprophyticus subsp. *saprophyticus*	16	16	0	100	5	11	31.3	15	1	93.8
S. simulans	9	2	7	22.2	1	8	11.1	0	9	0
S. capitis subsp. *urealyticus*	13	4	9	30.8	0	13	0	0	13	0
S. warneri	23	10	13	43.5	2	21	8.7	2	21	8.7
S. xylosus	20	17	3	85	0	20	0	3	17	15
Total	198	109	89	55.1	32	166	16.2	50	148	25.3

Results

The results of assessing methicillin-resistance by the individual tests in 12 species of staphylococci are shown in Table 1. Of the 198 isolates, 109 isolates (55.1%) were found to be resistant by the diffuse test with oxacillin and 32 isolates (16.2%) by the cefoxitin test. Based on the oxacillin MIC, resistance to methicillin was detected in 50 strains (25.3%). The above-mentioned results suggest a considerable variability of oxacillin-resistant isolates, ranging from 16.2% to 55.1%, depending on the phenotyping method used and recommended interpretation criteria.

Evaluation of the results of individual tests used for determining the resistance to oxacillin is shown in Table 2. The *mecA* gene was detected in 22 strains (11.1%), of which 15 were *S. epidermidis*, four *S. capitis* subsp. *urealyticus* and three *S. warneri*. The method for detecting the resistance to oxacillin/methicillin on the basis of PBP2a detection was 100% consistent with the *mecA* gene detection. Disk diffusion tests with both oxacillin and cefoxitin as well as the microdilution test with oxacillin MIC determination yielded both false-susceptible and false-resistant results. No resistant isolates were incorrectly identified as susceptible using the disk diffusion method with oxacillin (sensitivity of 100%). However, in addition to 22 correctly identified resistant isolates, another 87 isolates were incorrectly identified as resistant (a minor error; specificity of 50.6%). The other test, with cefoxitin, yielded the lowest sensitivity in determination of resistant isolates (45.5%). This means that 12 resistant isolates were incorrectly classified as susceptible (a major error). On the other hand, of the compared tests, this test was the best at classifying resistant isolates (specificity of 87.5%).

The results of determining MIC to oxacillin for the individual staphylococcal species are given in Table 3. A total of 50 isolates were classified as resistant (R) as their MIC was ≥ 0.5 mg/l. The species

Table 2. Analysis of the accordance and divergence (n isolates) in classification of 198 *Staphylococcus* spp. isolates as resistant (R) or susceptible (S) by the compared tests with reference *mecA* gene detection

Species	n	Compared tests													
		mecA	PBP2a	Oxacillin disk				Cefoxitin disk				Oxacillin MIC			
				accordance[a]		false		accordance[a]		false		accordance[a]		false	
				R	S	R	S	R	S	R	S	R	S	R	S
S. auricularis	13	0	0	0	3	10	0	0	12	1	0	0	12	1	0
S. saprophyticus subsp. *bovis*	17	0	0	0	2	15	0	0	15	2	0	0	5	12	0
S. epidermidis	20	15	15	15	4	1	0	10	4	1	5	15	4	1	0
S. haemolyticus	19	0	0	0	14	5	0	0	10	9	0	0	19	0	0
S. hominis subsp. *hominis*	10	0	0	0	8	2	0	0	10	0	0	0	10	0	0
S. hyicus	17	0	0	0	17	0	0	0	17	0	0	0	17	0	0
S. chromogenes	21	0	0	0	9	12	0	0	20	1	0	0	20	1	0
S. saprophyticus subsp. *saprophyticus*	16	0	0	0	0	16	0	0	11	5	0	0	1	15	0
S. simulans	9	0	0	0	7	2	0	0	8	1	0	0	9	0	0
S. capitis subsp. *urealyticus*	13	4	4	4	9	0	0	0	9	0	4	0	9	0	4
S. warneri	23	3	3	3	13	7	0	0	18	2	3	0	18	2	3
S. xylosus	20	0	0	0	3	17	0	0	20	0	0	0	17	3	0
Total	198	22	22	22	89	87	0	10	154	22	12	15	141	35	7

MIC – minimum inhibitory concentration; accordance[a] – accordance in classification of isolates as susceptible (S) or resistant (R) with the reference method of the *mecA* gene detection

Table 3. Oxacillin MIC values in isolates of 198 coagulase-negative staphylococci from raw materials and foodstuffs of animal origin

Species	No. of isolates	Oxacillin MIC (mg/l)				
		≤ 0.125	0.25	0.5	1	2
S. auricularis	13	-	12	1	-	-
S. saprophyticus subsp. *bovis*	17	-	5	9	3	-
S. epidermidis	20	-	4	-	7	9
S. haemolyticus	19	-	19	-	-	-
S. hominis subsp. *hominis*	10	-	10	-	-	-
S. hyicus	17	-	17	-	-	-
S. chromogenes	21	18	2	1	-	-
S. saprophyticus subsp. *saprophyticus*	16	-	1	13	2	-
S. simulans	9	9	-	-	-	-
S. capitis subsp. *urealyticus*	13	13	-	-	-	-
S. warneri	23	9	12	1	-	1
S. xylosus	20	8	9	3	-	-
Total	198	57	91	28	12	10

were as follows: *S. epidermidis* (32%), *S. saprophyticus* subsp. *saprophyticus* (30%), *S. saprophyticus* subsp. *bovis* (24%), *S. xylosus* (6%), *S. warneri* (4%), *S. chromogenes* (2%) and *S. auricularis* (2%). With the microdilution method, resistant isolates were classified with 68.2% sensitivity and 80.1% specificity; however, the method also produced minor (35 isolates) and major errors (7 isolates).

In the case of *S. epidermidis* isolates, the presence of PBP2a and the *mecA* gene was detected in 15 (93.8%) out of 16 isolates with oxacillin MIC ≥ 0.5 mg/l. As for the other coagulase-negative staphylococcal species with oxacillin MIC ≥ 0.5 mg/l (34 isolates), the presence of PBP2a was not detected in any of them. By contrast, the presence of PBP2a was detected in 7 isolates with oxacillin MIC ranging from 0.125 to 0.25 mg/l (four isolates of *S. capitis subsp. urealyticus* and three of *S. warneri*).

Discussion

The breakpoint or cut-off values for classification of methicillin-resistant staphylococci are crucial values to assessing this type of resistance. The CLSI recommendations (CLSI 2007) state the values of 2 mg/l for *S. aureus* strains and 0.25 mg/l for coagulase-negative

staphylococci (except for *S. lugdunensis*). The recommendations are supported by the study by Hájek et al. (2002) who reported that 29% of *S. epidermidis* isolates with MIC to oxacillin of 2 mg/l and classified as susceptible according to the accepted criteria (MIC R ≥ 4 mg/l) were carriers of the *mecA* gene. The oxacillin breakpoint value of 0.25 mg/l for coagulase-negative staphylococci was recommended by Tenover et al. (1999). They discovered that with the breakpoint of 2 mg/l, 38-74% of *mecA*-positive strains (depending on the media used) were incorrectly classified as susceptible whereas with the breakpoint of 0.25 mg/l, classification errors were in the range of 6-16% only. A the same time, however, the authors warn of potential false resistance in *mecA*-negative strains of certain species, in particular *S. warneri*, *S. capitis*, *S. lugdunensis* and *S. saprophyticus*.

This study suggests that 100% of coagulase-negative staphylococci (with the exception of *S. epidermidis* isolates) of animal origin with the oxacillin MIC range of 0.5-2 mg/l were incorrectly classified as resistant. By contrast, in *S. epidermidis* isolates with the above-mentioned oxacillin MIC range, the presence of the *mecA* gene and PBP2a was detected in 94%. Therefore, it is difficult to define a single MIC value as a precise criterion of resistance for all coagulase-negative staphylococci. For human isolates (except for *S. lugdunensis*), a single MIC value is recommended as the most appropriate, namely 0.25 mg/l (CLSI 2007). In such assessment, a major error, i.e. resistant isolates being incorrectly classified as susceptible, is considered unacceptable. Hussain et al. (2000) reported that in the most frequent human isolates of coagulase-negative staphylococci, such as *S. epidermidis*, *S. haemolyticus* and *S. hominis*, oxacillin MIC was ≥ 0.5 mg/l in cases of positive detection of the *mecA* gene. However, our data show that in coagulase-negative staphylococci of animal origin, the value of 0.25 mg/l is only valid for *S. epidermidis*. For the other species, 1 mg/l appears to be more appropriate. When using this value, only 1 out of 178 (1%) coagulase-negative staphylococci, with the exception of *S. epidermidis*, were false-resistant and 7 strains (4%) were false-susceptible.

Of the compared conventional phenotype tests for identification of methicillin-resistant isolates of coagulase-negative staphylococci, with respect to identification of resistant isolates (specificity of 87.5%), the screening method using a cefoxitin disk is viewed as the most appropriate test. It must be mentioned that 22 strains from the group of 198 strains were classified as false-resistant and 12 isolates as false-susceptible. Swenson et al. (2005) also reported false-susceptible and false-resistant isolates in the case of coagulase-negative staphylococci and the test with cefoxitin. The question is whether, in the case of coagulase-negative staphylococci of animal origin (with the exception of *S. epidermidis*), the size of cefoxitin zone used for determination of resistance should also be adjusted.

The study results suggest that also in veterinary practice it is advisable, when indicated, to confirm resistance to oxacillin, e.g. using PBP2a detection by latex agglutination. Although the method was primarily used to detect methicillin-resistance of *S. aureus*, it could be used for coagulase-negative staphylococci as well (Louie et al. 2001). This fact was confirmed by our study. In all strains of coagulase-negative staphylococci with the positively detected *mecA* gene, the test for the presence of PBP2a was also positive.

Rezistence k methicilinu u koaguláza-negativních stafylokoků a její detekce

Rezistence stafylokoků k methicilinu je významná především v případě izolátů *Staphylococcus aureus*, u koaguláza-negativních stafylokoků není její dopad ve veterinární medicíně přesně specifikován, tyto kmeny však mohou představovat důležitý rezervoár genů rezistence. Cílem předložené práce bylo stanovit rezistenci koaguláza-negativních stafylokoků izolovaných z potravin a surovin živočišného původu (mléko a mléčné výrobky) k oxacilinu a zhodnotit možnosti detekce této rezistence.

Celkem bylo testováno 198 koaguláza-negativních stafylokoků. Stanovení rezistence k methicilinu bylo provedeno difuzní diskovou metodou za použití disku oxacilinu a cefoxitinu, diluční mikrometodou, průkazem PBP2a a genu *mecA*. Ze 198 testovaných izolátů bylo v difuzním testu s oxacilinem určeno jako rezistentní 109 izolátů (55,1 %), v testu s cefoxitinem 32 izolátů (16,2 %) a podle minimální inhibiční koncentrace oxacilinu 50 izolátů (25,3 %). Žádný rezistentní izolát nebyl mylně identifikován jako citlivý diskovou difuzní metodou s oxacillinem (senzitivita 100 %). Avšak kromě 22 správně klasifikovaných rezistentních izolátů bylo dalších 87 izolátů mylně identifikováno rovněž jako rezistentní (specificita 50,6 %). Test s cefoxitinem vykazoval nejnižší senzitivitu stanovení rezistentních izolátů (45,5 %). Naopak rezistentní izoláty byly tímto testem klasifikovány nejsprávněji (specificita 87,5 %). Za použití mikrodiluční metody byly rezistentní izoláty klasifikovány se senzitivitou 68,2 % a specificitou 80,1 %.

Z výsledků vyplynula značná variabilita v detekci methicilin-rezistentních izolátů v závislosti na použité fenotypové metodě a doporučených interpretačních kritériích, a to od 16,2 % do 55,1 %. Je tedy zřejmé, že v případě koaguláza-negativních stafylokoků animální provenience (s výjimkou *S. epidermidis*) je vhodné zvážit stávající interpretační kritéria.

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