

DETECTION OF PBP2a (PENICILLIN-BINDING PROTEIN 2a) AND *mecA* GENE IN METHICILLIN RESISTANT STAPHYLOCOCCI ORIGINATED FROM ANIMALS

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*For the purpose of detecting methicillin (oxacillin) resistance in staphylococcal strains, in a number of microbiological laboratories only disc diffusion method with cefoxitin and/or oxacillin discs is used. Besides this method, it is desirable to determine MIC values for cefoxitin and/or oxacillin. After examination by disc diffusion and dilution methods, latex agglutination is used for the detection of PBP2a and PCR is used for the detection of *mecA* gene. Use of PCR is not possible in a large number of diagnostic laboratories and as method of choice, latex agglutination test for rapid detection of PBP2a is recommended. In this investigation, as confirmatory methods, latex agglutination and PCR were used for strains that were resistant to oxacillin and/or cefoxitin by disc diffusion and broth microdilution methods. In total, 14 strains of coagulase-negative staphylococci originating from clinical specimens of cats, dogs and chicken were examined. Among isolated strains, it was established that the dominating species was *Staphylococcus haemolyticus* with 11 isolated strains. Other isolated species were *Staphylococcus epidermidis*, *Staphylococcus capitis* and *Staphylococcus vitulinus*, each with one isolated strain.*

*For all 14 strains, oxacillin MIC values ranged from 0.5 µg/mL to >64 µg/mL and cefoxitin MIC values ranged from 1 µg/mL to >256 µg/mL. Positive agglutination reaction by latex agglutination test was recorded in 13 out of 14 strains. The PCR assay for *mecA* gene was positive in 12 investigated strains.*

Key words: latex agglutination, methicillin resistance, PCR, staphylococci

INTRODUCTION

Shortly after introduction of antibiotics in clinical practice, problem of antibiotic resistance emerged. Since then, this problem has raised concern in the entire world, in the scientific community, but also in the general public. Resistance

to all classes of antibiotics has been recorded and there are a limited number of new antibiotics available for the treatment of infections caused by multiresistance strains. Those facts are very disturbing and mankind is on the threshold of a new post antibiotic era.

For decades, strains of methicillin-resistant staphylococci (MRS) were presented as an important clinical and epidemiological problem in human medicine. Resistance to penicillinase stable penicillins has been marked as methicillin or oxacillin resistance. At one time methicillin was the surrogate for detecting resistance in staphylococci, but at one point it was replaced with oxacillin and later with ceftiofur as preferable agents for the detection of methicillin resistance in staphylococci. However the abbreviation MRS remained. The most important pathogen in genus *Staphylococcus* is *Staphylococcus aureus*, and there is a large number of data on methicillin resistance in this species. That is the reason why the current definition of methicillin-resistant staphylococci has been given by definition of MRSA (methicillin-resistant *S. aureus*). Methicillin-resistant *Staphylococcus aureus* strains (MRSA) are those strains of *S. aureus* that express *mecA* or another mechanism of methicillin resistance, such as changes in affinity of penicillin binding proteins for oxacillin (modified *S. aureus* (MODSA) strains) (Clinical and Laboratory Standard Institute-CLSI, 2010). In methicillin-resistant staphylococci the main mechanism of resistance to methicillin is mediated by *mecA* gene that is located on a mobile genetic element called the staphylococcal cassette chromosome (SCC) (Hanssen *et al.*, 2004; Zhang *et al.*, 2009). The gene *mecA* encodes production of PBP2a, the penicillin-binding protein that has no affinity for oxacillin. Those strains besides their resistance to oxacillin (methicillin) are resistant to all antibiotics belonging to the β -lactam class, including β -lactam/ β -lactamase inhibitors combinations, cepheims (with the exception of the newer cephalosporins with anti-MRSA activity) and carbapenems (CLSI, 2010). In a large number of cases, MRS strains, besides the listed antibiotics, are resistant to aminoglycosides, fluoroquinolones, tetracyclines, macrolides and chloramphenicol. That is the reason why a large number of infections due to those strains have a fatal outcome. Thus, besides types of resistance listed above, there are different mechanisms of methicillin resistance in staphylococci, such as hyperproduction of penicillinase and production of methicillinase, but these types of resistance are not so dangerous because those strains are, in general, susceptible to other classes of antibiotics (non- β -lactams).

Although there are many publication about MRSA and their significance, MRSA strains are not the only that are important. For a long time, CoNS (coagulase-negative staphylococci) have been considered as apathogenic and even if they were isolated from sterile body sites in pure cultures, they were declared as contaminants (Piette and Verschraegen, 2009). Nowadays, CoNS are getting more attention, because up to 80% of CoNS strains are methicillin resistant (Hanssen and Ericson Sollid, 2006; Piette and Verschraegen, 2009; Zhang *et al.*, 2009) and these strains are one of the most frequently isolated in clinical microbiology laboratories. It has been considered that CoNS represent a pool of resistance genes for *S. aureus* and other Gram-positive organisms (Hanssen and Ericson Sollid, 2006).

Until the beginning of the XXI century, the reports on the presence of MRS strains in animals were rare and mostly they were related to detect of MRS strains in the milk of healthy cows and in the milk of cows suffering from mastitis, as well (van Duijkeren *et al.*, 2004a). However, in the last few years, there has been an increasing number of publications which have been showing a significant presence of MRS strains on the skin and mucosal membranes in healthy horses (Busscher *et al.*, 2006), poultry, dogs and cats (van Duijkeren *et al.*, 2004a; Weese *et al.*, 2006). Since the animals - carriers of MRS strains are potentially dangerous reservoir for humans and, because the transmission of MRSA strains from human to animal (van Duijkeren *et al.*, 2004b; Rutland *et al.*, 2009), and vice versa was recorded, there is a need for rapid and precise detection of these strains in animals. The animals, pet animals as well as food animals, represent a very important reservoir of different bacterial species for humans. Pet animals are in a close contact with their owners and they share the same environment, which leads to exchange of microorganisms between animals, humans and environment.

The methods that are used in routine microbiological diagnostic, such as disc diffusion method, are not suitable for a definitive detection of MRS strains, thus these methods are useful only for bringing doubt. Because of that, the results obtained by disc diffusion need to be checked by broth or agar dilution methods, that are used to determine MIC (minimum inhibitory concentration) values of oxacillin and/or cefoxitin. For definitive identification of staphylococcal strains as MRS, it is necessary to demonstrate the presence of *mecA* gene or PBP2a. The presence of *mecA* gene can be precisely determined by using PCR assay with primers for *mecA*, except for novel *mecA* homologue, *mecA*_{LGA251} (García-Álvarez *et al.*, 2011). However, for now, PCR assay can be carried out only in reference laboratories, because it is technically demanding and expensive. Opposed to PCR, latex agglutination test can be used in routine microbiological diagnostics.

MATERIAL AND METHODS

Staphylococcal strains were isolated from clinical samples obtained from pet and domestic animals, which have been delivered to the Department of Microbiology, Faculty of Veterinary Medicine in Belgrade for routine diagnostic.

For isolation of investigated strains Columbia agar with 5% sheep blood (bioMérieux, France), MacConkey agar (bioMérieux, France) and nutrient broth (BioLab) were used. The commercial systems, API Staph (bioMérieux, France), ID32 STAPH (bioMérieux, France) and BBL Crystal Gram-Positive ID Kit (Becton Dickinson, USA) were used for identification of strains. The ability of investigated strains to produce coagulase was determined by the tube coagulase test (Veterinary Medicine Institute Zemun, Serbia).

The antibiogram discs of oxacillin (1 µg) and cefoxitin (30 µg) (Becton Dickinson, USA), as well as 100% pure active substances of oxacillin and cefoxitin (Sigma, USA) were used for the detection of methicillin-resistant staphylococci. Media for disc diffusion and broth microdilution were Mueller Hinton agar (bioMérieux, France) and Cation Adjusted Mueller Hinton Broth (Becton

Dickinson, USA). For oxacillin, 2% NaCl was added in Cation Adjusted Mueller Hinton Broth. Broth microdilution method was performed in U-bottom microtiter plates (Spekter, Čačak, Serbia). The reference strain *Staphylococcus aureus* ATCC 25923 was used for quality control of disc diffusion and the reference strain *Staphylococcus aureus* ATCC 29213 was used for quality control of broth microdilution method.

Susceptibility of isolated strains to oxacillin and cefoxitin was investigated by using disc diffusion and the obtained results were interpreted according to 2008 and 2010 CLSI recommendations. The inoculum density of investigated strains was approximately $1-2 \times 10^8$ CFU/mL, equivalent to 0.5 McFarland standard.

According to 2003 CLSI recommendations, broth microdilution method was performed and obtained MIC values for oxacillin and cefoxitin were interpreted according to 2008 and 2010 CLSI recommendations. The first step was to prepare a suspension with a density which corresponded to 0.5 McFarland standard ($1-2 \times 10^8$ CFU/mL). The suspension was diluted 10 times ($1-2 \times 10^7$ CFU/mL) in sterile saline and 0.005 mL of this suspension was inoculated in 0.1 mL of CAMHB. The final inoculum density was approximately 5×10^5 CFU/mL. For investigation purposes, oxacillin was prepared at concentrations ranging from 0.03 µg/mL to 64 µg/mL and cefoxitin at concentrations ranging from 0.12 µg/mL to 256 µg/mL. The solvent for both antibiotics was sterile distilled water. The MIC values for oxacillin and cefoxitin were recorded as the lowest concentrations of oxacillin and cefoxitin that completely inhibited growth of bacteria in a well.

The presence of PBP2a in staphylococci was detected using latex agglutination Slidex®MRSA Detection test (bioMérieux, France) according to manufacturer's instructions. This method was developed for detection of PBP2a in methicillin-resistant *S. aureus*. The reference strains methicillin-resistant *Staphylococcus aureus* ATCC 33591 and *Staphylococcus aureus* ATCC 25923 were used as quality controls.

The gold standard, PCR assay was used for detection of *mecA* gene. For extraction of DNA, a commercial kit for extraction of bacterial genomic DNA (Metabion, Germany) was used according to manufacturer's instructions. In the used protocol (Murakami *et al.*, 1993), *mecA* gene positive strains gave 533 bp PCR products. The primers (Invitrogen, USA) for amplification of 533 bp region of *mecA* gene had the following sequences: primer 1 (5'-AAA ATC GAT GGT AAA GGT TGG C-3') and primer 2 (5'-AGT TCT GCA GTA CCG GAT TTG C-3') each at 0.25 µM in the final concentration. Each of deoxyribonucleoside triphosphates (Fermentas) was used in a concentration of 200 µM. Other reagents and their concentrations were: PCR buffer (50 mM KCl), 1.5mM MgCl₂ and 5U/µL Taq DNA Polymerase (Fermentas). The amount of template DNA was 10 µL. The cycling conditions were: an initial denaturation at 94°C for 5 minutes and then 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, then a final extension at 72°C for 5 minutes in Autorisierter Thermocycler (Eppendorf, Germany). The visualization of PCR products was conducted on 1.5 % agarose gels in 1xTBE buffer by electrophoresis. The reference strains, methicillin-resistant *Staphylococcus aureus* ATCC 43300 was used as positive control and *Staphylococcus aureus* ATCC 25923 was used as negative control.

RESULTS AND DISCUSSION

Of the 14 analyzed strains of staphylococci, 11 were identified as *S. haemolyticus* and the remaining 3 strains were identified as *S. capitis*, *S. epidermidis* and *S. vitulinus*. All investigated strains were classified as resistant to oxacillin and/or ceftiofur by disc diffusion method. For all isolates of *S. haemolyticus* oxacillin MIC values were in the range from 2 µg/mL to ≥64 µg/mL and ceftiofur MIC values were in the range from 4 µg/mL to >256 µg/mL. For *S. capitis* MIC values for both antibiotics were 32 µg/mL. For *S. vitulinus* oxacillin MIC value was 0.5 µg/mL and ceftiofur MIC value was 1 µg/mL. For *S. epidermidis*



Figure 1. Positive latex agglutination reaction of one *Staphylococcus haemolyticus* strain (R1) and negative control of the same strain; quality control-positive latex agglutination reaction of MRSA ATCC 33591 and negative control of the same strain

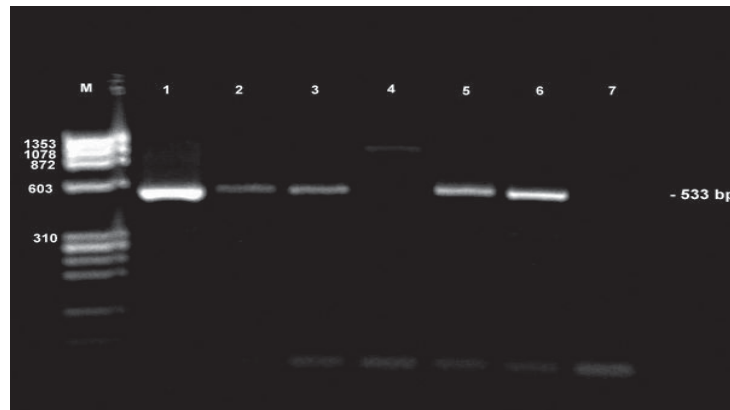


Figure 2. Agarose gel electrophoresis of amplified 533-bp region of *mecA*: M-ΦX174 DNA/BsuRI (HaeIII) Marker, 9 (Fermentas); lanes 1-3 *mecA* positive *S. haemolyticus* strains; lane 4 *mecA* negative *S. haemolyticus* strain; lane 5 *mecA* positive *S. capitis* strain; lane 6 MRSA ATCC 43300 (positive control) and lane 7 *S. aureus* ATCC 25923 (negative control)

oxacillin MIC value was 8 µg/mL and cefoxitin MIC value was 64 µg/mL. In 13 strains the presence of PBP2a was detected by Slidex®MRSA detection test (Figure 1). Only *S. vitulinus* gave a negative latex agglutination reaction. Among 14 isolates, 12 isolates were *mecA* positive (Figure 2). One of *S. haemolyticus* and *S. vitulinus* isolates were *mecA* negative (Table 1).

Table 1. Origin of the samples and isolated staphylococcal species, MIC values for oxacillin and cefoxitin, results of latex agglutination and presence of *mecA* gene

No.	Isolated species of staphylococci	Origin of the samples	Oxacillin MIC values (µg/mL)	Cefoxitin MIC values (µg/mL)	LATEX MRSA (bioMerieux)	PCR <i>mecA</i> gene
1	<i>Staphylococcus haemolyticus</i>	Ear swab of dog	64	16	+	+
2	<i>Staphylococcus haemolyticus</i>	Nasal swab of dog	>64	64	+	+
3	<i>Staphylococcus haemolyticus</i>	Wound swab of dog	>64	64	+	+
4	<i>Staphylococcus haemolyticus</i>	Eye swab of dog	2	4	+	-
5	<i>Staphylococcus capitis</i>	Ear swab of dog	32	32	+	+
6	<i>Staphylococcus vitulinus</i>	Throat swab of chicken	0.5	1	-	-
7	<i>Staphylococcus haemolyticus</i>	Wound swab of cat	>64	64	+	+
8	<i>Staphylococcus haemolyticus</i>	Eye swab of cat	>64	32	+	+
9	<i>Staphylococcus haemolyticus</i>	Skin swab of cat	>64	32	+	+
10	<i>Staphylococcus haemolyticus</i>	Ear swab of dog	64	8	+	+
11	<i>Staphylococcus haemolyticus</i>	Ear swab of dog	>64	64	+	+
12	<i>Staphylococcus epidermidis</i>	Dog urine	8	64	+	+
13	<i>Staphylococcus haemolyticus</i>	Nasal swab of cat	>64	>256	+	+
14	<i>Staphylococcus haemolyticus</i>	Wound swab of dog	>64	>256	+	+

There are many problems in the detection of methicillin resistance in CoNS (Stepanović *et al.*, 2006). One of the problems is heterogeneous resistance (Chambers, 1988; Hussain *et al.*, 2000; Ferreira *et al.*, 2003) that is more present in

CoNS strains than in MRSA strains (Yamazumi et al., 2001). Also, MIC values were established for *S. epidermidis* strains and a majority of non-*S. epidermidis* strains according to established interpretive criteria would have oxacillin MIC values $\geq 0.5 \mu\text{g/mL}$ (Tenover et al., 1999), but *mecA* gene would not be detected in all oxacillin-resistant strains. Further testing is recommended for CoNS strains isolated from serious infections for which oxacillin MIC values range from $0.5 \mu\text{g/mL}$ to $2 \mu\text{g/mL}$ (CLSI, 2010). Cefoxitin is considered to be a better predictor of methicillin resistance in staphylococci, but up to now (CLSI 2011) interpretive criteria for cefoxitin MIC values in CoNS have not been established, and in this investigation cefoxitin MIC values were interpreted according to breakpoints for *S. aureus* and *S. lugdunensis*. One strain of *S. haemolyticus* and *S. vitulinus* strain were *mecA* negative. Both strains were susceptible to cefoxitin and cefoxitin MIC values for those strains were $4 \mu\text{g/mL}$ and $1 \mu\text{g/mL}$, respectively. This corresponds to the current opinion that cefoxitin is better in predicting methicillin resistance in staphylococci, although this refers to the cefoxitin disc diffusion method (CLSI 2010). This finding was in agreement with other authors (Zhang et al., 2011).

For *S. haemolyticus* oxacillin MIC value was $2 \mu\text{g/mL}$ and for *S. vitulinus* $0.5 \mu\text{g/mL}$, so both strains were resistant to oxacillin by broth microdilution. Other authors (Stepanović et al., 2006) had similar results for *S. vitulinus*. Only one of 11 *S. haemolyticus* strains was *mecA* negative, which was similar to findings of other authors (Hussain et al., 2000) who classified staphylococci based on the presence of the *mecA* gene to four categories. *Staphylococcus haemolyticus* was included in category I, because 83.3% of strains were *mecA* positive with oxacillin MIC values $\geq 0.5 \mu\text{g/mL}$ and all *mecA* negative isolates had oxacillin MIC values $\leq 0.25 \mu\text{g/mL}$.

The latex agglutination test was negative in *S. vitulinus* that was also *mecA* negative. Some strains (4 strains of *S. haemolyticus*, *S. capitis* and *S. epidermidis*) gave positive latex agglutination reaction after 3 minutes (recommended agglutination reading time) and there were no correlation between oxacillin and cefoxitin MIC values and duration of agglutination reaction. There are reports that, induction of resistance with oxacillin (Ferreira et al., 2003), modification of the agglutination reaction time (Yamazumi et al., 2001) or modification of inoculum size (Louie et al., 2001) for CoNS increase the sensitivity of the test (Corso et al., 2004). The latex agglutination test is simple to perform and can give rapid results. The problem with this test is detection of PBP2a in CoNS. The manufacturer gave the remark that this test was made for the detection of PBP2a in *S. aureus*.

According to our results, it can be concluded that: 1) Disk diffusion with oxacillin and/or cefoxitin discs represented a preliminary method for the detection of methicillin resistance in staphylococci originated from animals; 2) Broth microdilution method with oxacillin was sensitive, but not specific for the detection of methicillin resistance in staphylococci originated from animals, because some strains of species other than *S. epidermidis* showed methicillin resistance, but those strains lacked *mecA* gene; 3) Slidex®MRSA Detection test was rapid and easy to perform, but for few strains modifications are needed for the improvement of sensitivity; 4) Classification of CoNS originated from animals as methicillin

resistant was not possible on the basis of one phenotypic test; 5) Cefoxitin broth microdilution was the only phenotypic method that gave results which matched 100% with PCR results.

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OTKRIVANJE PRISUSTVA PBP2a (PENICILLIN-BINDING PROTEINA 2a) I *mecA* GENA KOD METICILIN REZISTENTNIH STAFILOKOKA POREKLOM OD ŽIVOTINJA

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SADRŽAJ

Radi otkrivanja meticilin (oksacilin)-rezistentnih sojeva stafilocoka u većini mikrobioloških laboratorija koristi se najčešće samo disk difuziona metoda uz primenu antibiogram diskova oksacilina i cefoksitina. Pored navedene metode, poželjno je da se utvrde i vrednosti MIC oksacilina i/ili cefoksitina primenom dilu-

cione metode u bujonu ili agaru. Nakon ispitivanja pomenutim metodama za definitivno utvrđivanje pripadnosti soja grupi meticilin rezistentnih stafilokoka, koriste se metoda lateks aglutinacije za utvrđivanje prisustva PBP2a i PCR metoda za detekciju *mecA* gena. Kako primena metode PCR nije moguća u većini laboratorija koje se bave rutinskom dijagnostikom, kao metoda izbora preporučuje se lateks aglutinacioni test za brzo otkrivanje PBP2a. U ovom ispitivanju primenjeni su lateks aglutinacioni test i PCR metod, kao potvrđne metode za ispitivanje sojeva stafilokoka koji su bili rezistentni na oksacilin i/ili cefoksitin na osnovu rezultata dobijenih disk difuzionom metodom i mikrodilucionom metodom u bujonu. Ukupno je ispitano 14 sojeva koagulaza - negativnih stafilokoka izolovanih iz kliničkih uzoraka poreklom od pasa, mačaka i kokoši. Među izolovanim sojevima, ustanovljeno je da dominira vrsta *Staphylococcus haemolyticus* sa 11 izolata, a po jedan soj je izolovan od vrsta *Staphylococcus epidermidis*, *Staphylococcus capitis* i *Staphylococcus vitulinus*.

Vrednosti MIC oksacilina za 14 sojeva iznosile su od 0.5 mg/mL do >64 mg/mL, a vrednosti MIC cefoksitina iznosile su od 1 mg/mL do >256 mg/mL. Kod 13 od 14 sojeva utvrđena je pozitivna reakcija aglutinacije primenom lateks aglutinacionog testa. Kod 12 ispitivanih sojeva je utvrđeno prisustvo *mecA* gena PCR metodom.