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CHARACTERIZATION OF STAPHYLOCOCCAL CASSETTE CHROMOSOME *MEC* TYPES IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS INTERMEDIUS* STRAINS ISOLATED FROM DOGS

SCCmec types in mecA-positive S. intermedius strains were characterized by using multiplex PCR method described previously by Oliveira and Lencastre [11]. A total of 100 preliminary identified S. intermedius isolates were tested to determine their species identify and mecA gene encoding methicillin resistance. The duplex PCR assay was able to identity all the strains carring mecA gene (17%). The study showed that 4% strains identified as S. intermedius based upon their phenotypic properties do not yielded a 16S rRNA gene amplification product, indicating staphylococci other than S. intermedius. SCCmec type IIIC was identified in 15 strains (88%), type IB was found only in one strain. One strain was not typable by this technique.

K e y w o r d s: Staphylococcus intermedius, multiplex PCR, mecA gene, MRSI, SCCmec types.

Staphylococcus intermedius was first described as a new species in 1976 and was differentiated from *Staphylococcus aureus* and *Staphylococcus epidermidis* based on its biochemical and physiological characteristics [3]. This microorganism occurs as commensal bacteria on the skin and mucosal membranes of dogs. This species has also been found in cats, horses, pigeons, foxes, and other animals. In dogs it has been implicated in serious infections, such as: pyoderma, otitis externa, abscess, and infections of eyes, joints, mammary glands, respiratory tract and gastrointestinal tract [2]. In humans *S. intermedius* is recognized as an invasive zoonotic pathogen and has been isolated in 18% of canine-inflicted wounds [5, 8]. On the other hand it has been infrequently identified in other invasive human diseases and only a few studies have reported rare cases of infections such as bacteremia [14], infection of mastoid cavities [5], and brain abscesses [1].

Increased frequency of infections caused by *S. intermedius* exhibiting multi-drug resistance, including methicillin, is being observed among dogs [6, 9]. Methicillin resistance in staphylococci is mediated by the *mecA* gene, encoding the penicillin-binding protein 2a (PBP2a), which has reduced affinity for methicillin, oxacillin and other beta-lactam antibiotics. The *mecA* gene, responsible for this phenomenon

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has already been identified in *S. intermedius* strains isolated from dogs [6, 9]. Methicillin resistance is most often associated with a mobile genetic element termed the staphylococcal cassette chromosome *mec* (SCC*mec*). The molecular basis of methicillin resistance has been well characterized in *S. aureus*, but little is known about the acquisition and the organization of its genetic key, the *mecA* gene, in other staphylococcal species. In *S. aureus* several types and subtypes of Staphylococcal chromosome cassettes (SCC*mec*) have been classified on the basis of the different combinations of the two main parts: the *mec* gene complex, which encodes methicillin resistance (the *mecA* gene and its regulators), and the *ccr* gene complex, which encodes site-specific recombinases responsible for its mobility [7, 10].

In this study we developed a duplex PCR assay, which allows to conduct the simultaneous identification of *Staphylococcus intermedius* strains and the *mecA* gene detection. We also characterize SCC*mec* types in methicillin-resistant *Staphylococcus intermedius* strains isolated from dogs using multiplex PCR strategy.

Materials and methods

A total of 100 strains were isolated in the Diagnostic Laboratory of the Division of Bacteriology and Molecular Biology at Warsaw University of Life Sciences. The strains were cultivated from different clinical specimens taken from dogs. Strains used for control purpose include the: *Staphylococcus aureus* ATCC 6538, *Staphylococcus intermedius* ATCC 29663 and methicillin-resistant *Staphylococcus aureus* (MRSA) 14.002 (possessed from The National Reference Centre for Antimicrobial Susceptibility).

The isolates were identified by means of the API Staph system (BioMérieux) and additional characteristics such as type of hemolysis, colony pigment, coagulase, clumping factor and acid production from maltose. Sensitivity to methicillin was assessed using a disc diffusion test according to the Clinical Laboratory Standards Institute (CLSI), with discs containing 1 μg of oxacillin (BioMérieux). Oxacillin-resistant, *mecA*-positive strains were tested for resistance to other antimicrobial agents.

Bacterial cultures were grown overnight at 37 °C in 5 ml of brain-heart infusion broth (BioMérieux). Chromosomal DNA was obtained using DNA Genomic Mini (A&A Biotechnology). For DNA extraction 1.5 ml of bacterial culture was centrifugated at 6 000 g for 5 minutes. Sediment was suspended in 140 μl TE buffer with 2% glucose, containing 5 μl lysozyme (5 mg/ml, SIGMA), 2.5 μl lysostaphin (1 mg/ml, SIGMA) and 2 μl ribonuclease A (10 mg/ml, Fermentas). After incubation for 2 hours at 37 °C further part of the DNA isolation process was conduced according to the manufacturer's instructions.

Two pairs of primers were used for the detection of *mecA*-positive *Staphylococcus intermedius* strains. One was specific for *mecA* gene and one for *S. intermedius*-specific fragment of the 16S rRNA gene. The cycling conditions were as follows: after an initial denaturation step of 94 °C for 3 min, samples completed 30 cycles of amplification (30 s of denaturation at 94 °C, 30 s of annealing at 55 °C and 1.5 min of extention at 72 °C). The final elongation was performed at 72 °C for 4 min. The primer sequences, products size and references are presented in Table 1.

Table 1

Oligonucleotide primer sequences used in the duplex PCR

Primer	Gene amplified	Sequence	Product size (bp)	References
Inter1	16S rRNA	5'-CCGTATTAGCTAGTTGGTGG-3'	901	[15]
Inter2		5'-GAATGATGGCAACTAAGTTC-3'		
MecA1	mecA	5'-AAAATCGATGGTAAAGGTTGGC-3'	523	[13]
MecA2		5'-AGTTCTGCAGTACCGGATTTGC-3'		

The PCR products, 901 bp and 523 bp in size were separated on 1% agarose gels in Tris-Acetate-EDTA buffer, stained with ethidium bromide, visualized with UV light and analyzed using the VersaDoc Model 1000 Imaging System with Quantity One 4-4-0 software (BioRad). Typical electrophoresis pattern of *mecA* and 16S rRNA gene amplification product are presented on Fig. 1.

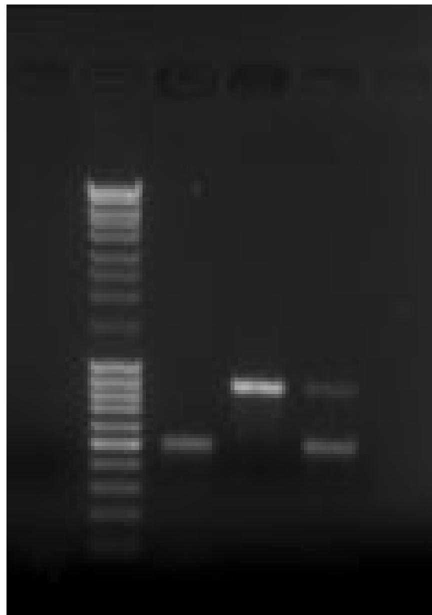

Fig.1. PCR for the species confirmation and detection of *mecA* gene
Lane 1 — negative control; lane 2 — MassRuler DNA Ladder Mix (Fermentas); lane 3 — *mecA* product for MRSA strain no. 14.002; lane 4 — 16S rRNA gene amplication product for *S. intermedius* ATCC 29663; lane 5 — one of the researched strains (MRSI).

The 17 *mecA*-positive *S. intermedius* strains were used to characterize the SCC*mec* types. SCC*mec* multiplex PCR typing assay was based on Oliveira's method [11]. Totally 9 pairs of the primers for SCC*mec* types and subtypes, as well as for the *mecA* gene were used (tab. 2).

Table 2

Oligonucleotide primer sequences used in the multiplex PCR

Locus	Primer	Oligonucleotide sequence (5'–3')	Product size (bp)	Specificity (SCC*mec* type)
A	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	I
	CIF2 R2	ATTTACCACAAGGACTACCAGC		
B	KDP F1	AATCATCTGCCATTGGTGATGC	284	II
	KDP R1	CGAATGAAGTGAAAGAAAGTGG		
C	MECI P2	ATCAAGACTTGCATTCAGGC	209	II, III
	MECI P3	GCGGTTTCAATTCACTTGTC		
D	DCS F2	CATCCTATGATAGCTTGGTC	342	I, II, IV
	DCS R1	CTAAATCATAGCCATGACCG		
E	RIF4 F3	GTGATTGTTCGAGATATGTGG	243	III
	RIF4 R9	CGCTTTATCTGTATCTATCGC		
F	RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	III
	RIF5 R13	GTCACAGTAATTCCATCAATGC		
G	IS431 P4	CAGGTCTCTTCAGATCTACG	381	
	pUB110 R1	GAGCCATAAACACCAATAGCC		
H	IS431 P4	CAGGTCTCTTCAGATCTACG	303	
	pT181 R1	GAAGAATGGGGAAAGCTTCAC		
mecA	MECA P4	TCCAGATTACAACTTCACCAGG	162	Internal control
	MECA P7	CCACTTCATATCTTGTAACG		

The cycling conditions were as follows: after an initial denaturation step of 94 °C for 4 min, samples completed 30 cycles of amplification (30 s of denaturation at 94 °C, 30 s of annealing at 53 °C and 1 min of extention at 72 °C). The final elongation was performed at 72 °C for 4 min.

The strains used for control purposes include the methicillin/oxacillin resistant *S. aureus* strains carrying: I, IA, IB, II, III, IIIA, IIIB, IIIC, IIID, IIIE, IIIG, IIIJ and IV type of cassette. The standard strains were obtained from National Medicines Institute, Division of Clinical Microbiology and Infection Prevention.

The PCR products were separated on 2% agarose gels in Tris-Acetate-EDTA buffer, stained with ethidium bromide, visualized with UV light and analyzed using a VersaDoc Model 1000 Imaging System with Quantity One 4-4-0 software (BioRad).

Results and discussion

All strains investigated in the present study were predominarily identified as *S. intermedius* based upon their phenotypic properties. In vitro antimicrobial susceptibility tests showed that 17 isolates were resistant to methicillin. Application of duplex PCR yielded following results as shown in Table 3.

Table 3

PCR testing results for the presence of *mecA* and 16S rRNA genes among 100 canine isolates preliminary identified as *S. intermedius*

Methicillin susceptibility phenotypes of tested strains determined by the disc diffusion method	Results of duplex PCR assay			
	***mecA* +** **16S rRNA +**	***mecA* –** **16S rRNA +**	***mecA* +** **16S rRNA –**	***mecA* –** **16S rRNA –**
Methicillin - resistant (n=17)	17 (17%)	0	0	0
Methicillin - susceptible (n=83)	0	79 (79%)	0	4 (4%)

The result of duplex PCR assay showed that 4% of the isolates were misidentified as *S. intermedius* upon the identification of the phenotypic test. Totally of 17% of strains recognised as *S. intermedius* carring *mecA* gene. For 79% of strains we obtained a species-specific product for the 16S rRNA gene of *S. intermedius* but no *mecA* product, as expected for methicillin-sensitive *Staphylococcus intermedius* (MSSI).

SCC*mec* type IIIC was identified in 15 *mecA*-positive strains (88%), type IB was found in one strain (6%). One isolate (6%) was not typable by this technique. The results of multiplex PCR of exemplary 7 investigated *S. intermedius* strains are presented on Fig. 2.

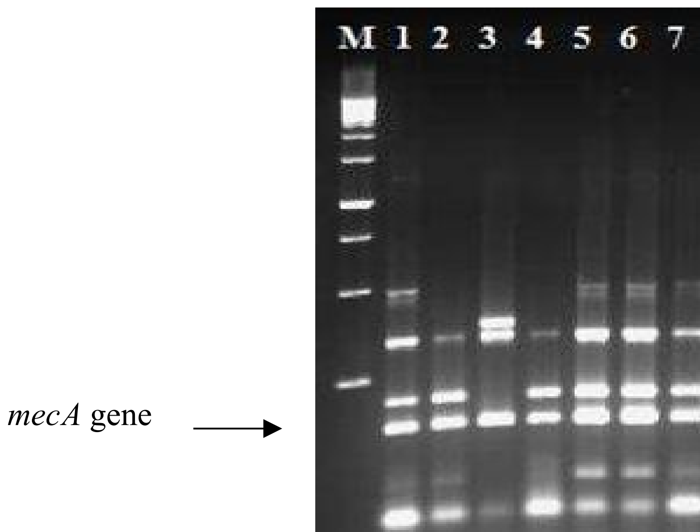

Fig. 2. PCR SCC*mec* profiles from methicillin-resistant *S. intermedius*
M — DNA molecular size marker; 1—7 — exemplary 7 investigated *S. intermedius* strains; SCC*mec* type IB — lanes 3; SCC*mec* type IIIC — lanes 1, 2 and 4 to 7.

The occurence of *S. intermedius* strains resistant to all antimicrobials commonly used in veterinary medicine is alarming. The results of recent studies have proved that some isolates are resistant to methicillin by expression of *mecA* gene and thus the abbreviation methicillin-resistant *Staphylococcus intermedius* (MRSI) is appropriate in analogy to MRSA. The worldwide increase in the number of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has emphasised the need for the fast and reliable identification and typing methods. In addition to genotyping characterization of the staphylococcal cassette chromosome (SCC) *mec* type has led to better discrimination of hospital-acquired MRSA (HA-MRSA), carrying one of three types of SCC*mec* (type I, II, or III) and nonmultiresistant community-acquired MRSA (CA-MRSA) carrying type IV or V SCC*mec*. Type IV and V SCC*mec* are small elements that do not carry the antibiotic resistance genes other than *mecA* and has the multiple subtypes [4]. Presence of this small (IV) type of SCC*mec* was observed in *S. schleiferi* subsp. *coagulans* strains isolated from companion animals [12]. Worthwhile to point out that SCC*mec* elements lacking *mecA* have also been reported in *S. aureus* and coagulase-negative staphylococci. SCCnon-*mec* is located at the same chromosomal site as all SCC*mec* elements, and it contains a virulence factor called capsular polysaccharide 1, which makes the strain more resistant to phagocytosis [10].

In this study we found that *S. intermedius* strains isolated from dogs possessed the type I and III SCC*mec*, and we observed that all these strains were resistant to all beta-lactams and also to other groups of antibiotics (aminoglycosides, thrimethoprim, sulfonamides, tetracyclines, macrolides, and fluorochinolones). Their MIC values were high (data not shown). One isolate was not typable by the method used in this study, and this may indicate that there are a variety of uncharacterized SCC*mec* elements in staphylococcal species other than *S. aureus*.

Little concern has been voiced yet about the possibility of animal to human *mecA* gene transmission.

Canine strains of *S. intermedius* have been found to harbor SCC*mec* elements homologues to those carried by *S. aureus*.

Our findings suggest that the high prevalence of this resistancy vector in dogs may be the reservoirs of antibiotic resistancy genes, and may perhaps be the driving force for the generation of new staphylococcal methicillin-resistant strains. The fact that dogs are in close contact with their owners, the risk of transmission of such bacteria between animals and humans must be considered.

Canine strains of *S. intermedius* have been found to harbor SCC*mec* elements encoding determinants for the expression of the methicillin-resistance phenotype. The SCC*mec* typing strategy we used in this study detected two SCC*mec* elements (SCC*mec* type IB and IIIC). One isolate was not typable by this method.

The diversity of SCC*mec* types found in dogs appears similar to that seen in humans, however uncharacterized SCC*mec* elements in *S. intermedius* may exist.

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ХАРАКТЕРИСТИКА СТАФИЛОКОККОВОЙ КАССЕТНОЙ ХРОМОСОМЫ *MEC* ТИПА У МЕТИЦИЛЛИН-РЕЗИСТЕНТНЫХ ШТАММОВ *STAPHYLOCOCCUS INTERMEDIUS,* ИЗОЛИРОВАННЫХ ОТ СОБАК

Реферат

SCC*mec* тип у *mecA*-положительных штаммов *S. intermedius* был охарактеризован с использованием метода мультиплексной ПЦР, описанного ранее Oliveira и Lencastre [11]. 100 предварительно идентифицированных изолятов *S. intermedius* были исследованы для подтверждения их видовой принадлежности и для обнаружения *mecA* гена, кодирующего резистентность к метициллину. Дуплексный ПЦР анализ дал возможность идентифицировать все штаммы, несущие *mecA* ген (17%). Исследования показали, что 4% штаммов, идентифицированных как *S. intermedius* на основании их фенотипических свойств, не давали продуктов амплификации гена 16S rРНК, определяющих стафилококки отличные от *S. intermedius*. SCC*mec* типа IIIC была обнаружена у 15 (88%) штаммов, типа IB — только у одного штамма. Лишь один штамм не поддавался типированию с помошью этого подхода.

К л ю ч е в ы е с л о в а: *Staphylococcus intermedius*, мультиплексная ПЦР, *mecA* ген, MRSI, типы SCC*mec*.

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ХАРАКТЕРИСТИКА СТАФІЛОКОКОВОЇ КАСЕТНОЇ ХРОМОСОМИ *MEC* ТИПУ У МЕТИЦИЛІН-РЕЗИСТЕНТНИХ ШТАМІВ *STAPHYLOCOCCUS INTERMEDIUS,* ИЗОЛЬОВАНИХ ВІД СОБАК

Реферат

SCC*mec* тип у *mecA*-позитивних штамів *S. intermedius* був охарактеризований з використанням метода мультиплексної ПЛР, що був описаний раніше Oliveira і Lencastre [11]. 100 попередньо ідентифікованих ізолятів *S. intermedius* були досліджені для підтвердження їх видової приналежності та для виявлення *mecA* гена, що кодує резистентність до метициліну. Дуплексний ПЛР аналіз дав можливість ідентифікувати усі штами, що несуть *mecA* ген (17%). Дослідження показали, що 4% штамів, ідентифікованих як *S. intermedius* на підставі їх фенотипових властивостей, не давали продуктів ампліфікації гена 16S rРНК, що виявляють стафілококи відмінні від *S. intermedius*. SCC*mec* типу IIIC була виявлена у 15 (88%) штамів, типу IB — тільки у одного штаму. Лише один штам не піддавався типуванню за допомогою даного підходу.

К л ю ч о в і с л о в а: *Staphylococcus intermedius*, мультиплексна ПЛР, *mecA* ген, MRSI, типи SCC*mec*.