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Staphylococcal cassette chromosome *mec* elements from *Staphylococcus intermedius* group (SIG) isolates from dogs in a center for veterinary diagnostics in Brazil

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ABSTRACT: *Methicillin resistance in the Staphylococcus intermedius group (SIG) has emerged in small animal practice. Methicillin-resistant SIG (MRSIG) members have been implicated as causes of infections in both companion animals and humans. Staphylococcal cassette chromosome mec (SCCmec) elements carry the mecA/C genes, which encode for the transpeptidase PBP2a (PBP2') responsible for β-lactam antibiotic resistance in staphylococci. This study examined the SCCmec types of MRSIG isolates from different clinical specimens of dogs that exhibited methicillin MIC ≥ 0.5 μg/mL by an automated identification and susceptibility system in a Center for Veterinary Diagnostics in São Paulo, Brazil. Susceptibility to methicillin was determined by broth microdilution testing, and Oxoid® M.I.C.Evaluator® strips. PBP2a production was detected using a latex agglutination assay. SCCmec typing was performed according to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) guidelines. SCCmec type II (2A), SCCmec type III (3A), composite SCC structures consisting of a class A mec gene complex in addition to multiple ccr gene complexes, and non-typable SCCmec elements were reported in these MRSIG isolates. SCCmec type variants differing from those so far acknowledged by IWG-SCC were found, indicating new rearrangements in the genetic context of mecA in these canine MRSIG isolates.*

Key words: *Staphylococcus intermedius group (SIG), methicillin resistance, SCCmec elements, companion animals.*

Cassetes cromossômicos estafilocócicos *mec* de isolados do grupo *Staphylococcus intermedius* (GSI) de cães em um centro veterinário de diagnósticos no Brasil

RESUMO: *A resistência à meticilina no grupo Staphylococcus intermedius (GSI) tem aumentado na clínica de pequenos animais. Membros GSI resistentes à meticilina (GSIRM) têm sido causas de infecções tanto em animais de companhia e humanos. Cassetes cromossômicos estafilocócicos mec (SCCmec) carregam os genes mecA/C, que codificam a transpeptidase PBP2a (PBP2') responsável pela resistência aos antibióticos β-lactâmicos em estafilococos. Nosso objetivo foi investigar os elementos SCCmec de GSIRM isolados de diferentes amostras clínicas de cães que exibiram CIM de meticilina ≥ 0,5 μg/mL por meio de um sistema automatizado em um Centro Veterinário de Diagnósticos em São Paulo, Brasil. A sensibilidade à meticilina foi determinada por meio do teste de microdiluição em caldo e fitas Oxoid® M.I.C.Evaluator®. A produção de PBP2a foi detectada usando um ensaio de aglutinação de látex. A tipagem dos elementos SCCmec foi realizada de acordo com as diretrizes do International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). SCCmec tipo II (2A), SCCmec tipo III (3A), SCC compostos de um complexo mec de classe A com múltiplos complexos ccr, e elementos SCCmec não tipáveis foram encontrados nesses isolados GSIRM. Variantes que diferem dos elementos SCCmec reconhecidos até o momento pelo IWG-SCC foram encontradas, indicando novos rearranjos no contexto genético de mecA nesses isolados GSIRM caninos.*

Palavras-chave: *Grupo Staphylococcus intermedius (GSI), resistência à meticilina, SCCmec, animais de companhia.*

The *Staphylococcus intermedius* group (SIG) includes *Staphylococcus intermedius*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini*, Gram-positive cocci well adapted to the skin and mucosal microbiomes of a variety of animal hosts (MURRAY et al., 2018; YARBROUGH et al., 2018). SIG isolates have emerged as leading causes of infection of the urinary

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and respiratory tracts, surgical wounds, ears and skin in companion animals, and have also been increasingly associated with non-bite-related injuries in humans (VAN DUIJKEREN et al., 2011; DAVIS et al., 2014; SOMAYAJI et al., 2016). Identification of coagulase-positive staphylococci by MALDI-TOF MS or molecular methods has reliably allowed SIG species differentiation that have since been underreported or commonly misidentified as *Staphylococcus aureus* by conventional methods in clinical microbiology laboratories. Antibiotic-resistant SIG infections have raised concern in both veterinary small-animal practice and humans, in particular methicillin-resistant *S. pseudintermedius* (MRSP) infections. MRSP is an opportunistic pathogen responsible for pyoderma, otitis, and wound infections in pets, and its transmission between animals and humans has been well documented (VAN DUIJKEREN et al., 2011; BÖRJESSON et al., 2012; BARDIAU et al., 2013; COUTO et al., 2014; GRÖNTHAL et al., 2014; MACCARTHY et al., 2015; DUIM et al., 2016; FEßLER et al., 2018; WORTHING et al., 2018).

Methicillin resistance in staphylococci occurs due to expression of the penicillin binding protein PBP2a (PBP2'), a transpeptidase which shows a slow rate of acylation by β-lactam antibiotics (FISHOVITZ et al., 2014). PBP2a is encoded by the *mecA* or *mecC* genes that are carried by mobile staphylococcal cassette chromosome *mec* (SCC*mec*) elements (HIRAMATSU et al., 2013). The SCC*mec* typing has long been used for understanding the epidemiology of methicillin-resistant *S. aureus* (MRSA) and community-associated MRSA (CA-MRSA) strains (CHAMBERS & DELEO, 2009), and it has also been useful to investigate MRSP spread that now has emerged as a zoonotic pathogen (PERRETEN et al., 2013; QUITOCO et al., 2013; MACCARTHY et al., 2015; WORTHING et al., 2018).

SCC*mec* type assignment has been established by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), and it is based on a combination of the *mec* gene complex (*mecA-C* resistance genes, *mecR1* and *mecI* regulatory genes, and insertion sequences), and the *ccr* gene complex (cassette chromosome recombinases *ccr*) (IGW-SCC, 2009; HIRAMATSU et al., 2013). The only few SCC*mec* elements so far characterized in MRSP indicated an evolutionary diversification of SCC*mec* structure in certain clonal lineages. Understanding how the genetic context of the *mec* gene has evolved in SIG isolates is crucial for controlling methicillin resistance that is on the rise in veterinary and healthcare settings. Therefore, in this study we examined the methicillin resistance phenotype and SCC*mec* types of methicillin-resistant SIG (MRSIG) isolates from different clinical specimens of dogs from a Center for Veterinary Diagnostics in São Paulo, Brazil.

From January to May 2014, a total of 41 SIG isolates exhibiting resistance to methicillin by BD PHOENIX Automated Microbiology System (MIC $\geq$ 0.5 µg/mL) were recovered from various canine specimens in a routine diagnostic laboratory in SP, Brazil (Table 1). Skin, eye and ear samples were obtained using sterile swabs, whilst urine and ascitic fluid samples were obtained by cystocentesis and abdominal puncture, respectively. Overnight cultures at 37 °C of these samples yielded colonies with a double zone of beta-hemolysis on blood agar (5% sheep blood), which were submitted to biochemical conventional methods used to differentiate coagulase-positive *Staphylococcus* species from *S. aureus*, such as the latex slide agglutination test (clumping factor/ protein A), the tube coagulase test, DNase production, PYR, and polymyxin B resistance (BECKER et al., 2015).

Susceptibility to methicillin was determined by broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute – CLSI (2018). Oxacillin susceptibility breakpoints added to CLSI supplement M100 were used to predict *mecA*-mediated oxacillin resistance (CLSI, 2016). Oxacillin M.I.C. evaluator strips (M.I.C.E., Thermo Fisher Scientific, Basingstoke, UK) were also used for all MRSIG isolates. *S. aureus* subsp. *aureus* ATCC 29213 was used as a control for antimicrobial susceptibility testing. The product of *mecA* was detected using the PBP2a latex agglutination assay (Oxoid, Hampshire, United Kingdom) according to the manufacturer's instructions. *S. aureus* ATCC 29213 (methicillin susceptible, *mecA* negative), and MRSA N315 (methicillin resistant, *mecA* positive) were used as controls for PBP2a production.

DNA of the 41 MRSIG isolates was extracted using DNeasy blood and tissue kits (Qiagen, USA), with lysozyme (50 mg/ml) and lysostaphin (10 mg/ml) being added to the initial DNA extraction step. The SCC*mec* typing was performed according to the IWG-SCC guidelines. PCR assays to identify *mec* classes and *ccr* types were performed using primer sets as previously described (KONDO et al., 2007; ITO et al., 2014). Then, SCC*mec* types were assigned based on a combination of the type of *mec* gene complex (A, B, C1 and C2) and the *ccr* gene allotype (1 to 5). The MRSA reference strains NCTC 10442 (type I), N315 (type II), 85/2082 (type III),

Table 1 - SCC*mec* elements identified in MRSIG isolates from clinical infections in dogs in Brazil.

Isolate	Sample	Oxacillin MIC (mg/L)	------------------*mec* gene complex------------------					-----------------*ccr* gene complex-------------					SCC*mec* type
			mecA	*mecC*	Class A	Class B	Class C	*ccrAB1*	*ccrAB2*	*ccrAB3*	*ccrAB4*	*ccrC*	
1	Skin	< 0.5	-	-	-	-	-	-	-	-	-	-	-
2	Skin	< 0.5	-	-	-	-	-	-	-	-	-	-	-
4	Skin	0.5	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
5	Skin	256	+	-	+	-	-	-	-	+	+	-	III/VIII
6	Skin	< 0.5	-	-	-	-	-	-	-	-	-	-	-
7	Skin	0.5	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
8	Skin	8	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
9	Skin	1	+	-	-	-	-	-	+	-	-	+	non-typable
10	Skin	< 0.5	+	-	-	-	-	-	+	-	-	+	non-typable
11	Skin	> 512	+	-	+	-	-	-	-	+	+	-	III/VIII
14	Skin	1	+	-	-	-	-	-	+	-	-	+	non-typable
15	Skin	> 512	+	-	+	-	-	-	-	+	+	-	III/VIII
16	Skin	< 0.5	-	-	-	-	-	-	-	-	-	-	-
17	Skin	< 0.5	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
19	Skin	> 512	+	-	+	-	-	-	-	+	+	-	III/VIII
21	Skin	> 512	+	-	+	-	-	-	-	+	+	-	III/VIII
23	Skin	512	+	-	+	-	-	-	-	+	-	-	III (3A)
24	Skin	256	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
25	Skin	512	+	-	+	-	-	-	-	+	+	+	III/VIII + *ccrC*
26	Skin	> 512	+	-	+	-	-	-	-	+	+	+	III/VIII + *ccrC*
31	Skin	256	+	-	-	-	-	-	+	-	-	+	non-typable
32	Skin	256	+	-	-	-	-	-	+	-	-	+	non-typable
33	Skin	128	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
34	Skin	512	+	-	+	-	-	-	+	+	+	+	II/III/VIII + *ccrC*
35	Skin	256	+	-	-	-	-	-	+	-	-	+	non-typable
36	Skin	512	+	-	+	-	-	-	+	+	-	-	II/III
37	Skin	512	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
38	Skin	> 512	+	-	+	-	-	-	-	+	+	+	III/VIII + *ccrC*
39	Skin	64	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
40	Skin	> 512	+	-	+	-	-	-	+	+	+	-	II/III/VIII
41	Skin	> 512	+	-	+	-	-	-	+	+	-	+	II/III + *ccrC*
12	Ear	> 512	+	-	+	-	-	-	-	+	+	-	III/VIII
20	Ear	2	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
28	Ear	16	+	-	+	-	-	-	+	+	-	+	II/III + *ccrC*
29	Ear	256	+	-	+	-	-	-	-	+	-	-	III (3A)
30	Ear	256	+	-	+	-	-	-	-	+	+	-	III/VIII
13	Urine	< 0.5	+	-	+	-	-	-	-	+	+	-	III/VIII
18	Urine	< 0.5	-	-	-	-	-	-	-	-	-	-	-
27	Urine	256	+	-	+	-	-	-	+	+	+	+	II/III/VIII + *ccrC*
3	Ascitic fluid	0.5	+	-	+	-	-	-	+	-	-	+	II (2A) + *ccrC*
22	Eye	512	+	-	+	-	-	-	-	+	+	-	III/VIII

JCSC1978 (type IV), and MRSA clinical strains from our laboratory collection (types V, VI, VII and VIII) were used as controls. Sanger sequencing was previously carried out to confirm all PCR amplicons from the MRSA control strains.

The canine MRSIG isolates of this study were carriers of SCC*mec* type II (2A) (10/41), SCC*mec* type III (3A) (2/41), and composite SCC structures consisting of a class A *mec* gene complex in addition to multiple *ccr* gene complexes as follows: types 2 and 3 (1/41); types 2, 3, and 4 (1/41); types 2, 3, and 5 (2/41); types 2, 3, 4, and 5 (2/41); types 3 and 4 (9/41) and types 3, 4, and 5 (3/41). *mecA*-positive MRSIG isolates carrying non-typable SCC*mec* elements were also reported (6/41). These SCC*mec* elements consisted of *ccr* gene complexes types 2 and 5, but classes of *mec* gene complex (A, B, C1 and C2) could not be identified using the primer sets described by KONDO et al. (2007) and Ito et al. (2014) (Table 1). Composite SCC structures have been reported by carrying an association of a *mec* gene complex with two or more *ccr* gene allotypes, which may result from deletions of the original J region or insertions of other IS elements (CHANCHAITHONG et al., 2015). Genetic organization of the new composite SCC structures, such as SCC*mec* II-III, SCC*mec* VII-241, SCC*mec* V, ψSCC*mec*57395, SCC*mec* IVg, SCC*mec* VT, and SCC*mec*KW21 has been characterized in MRSP, and consist of unusual combinations of *mec* gene complexes with *ccr* gene complexes (DESCLOUX et al., 2008; BLACK et al., 2009; SHORE et al., 2011; PERRETEN et al., 2013; CHANCHAITHONG et al., 2015; WU et al., 2015; DUIM et al., 2018).

All MRSIG isolates harboring SCC*mec* type II (2A) also carried a *ccrC* gene, in addition to the *ccrA2B2* gene, but the class A *mec* gene complex was disrupted in five of these SCC*mec* elements. The primer set constructed on *mecA* and *mecI* produced a PCR amplicon smaller than the expected size. Then, other primers to amplify internal fragments of *mecA* and its regulatory genes *mecR1* and *mecI* were used indicating that *mecR1* was disrupted. Class A *mec* is considered the prototype complex that contains the *mecA* regulatory locus. Disruption of the signal transducer protein MecR1 did not affect the *mecA* expression, as high oxacillin MIC levels (128 - 512 μg/mL) were exhibited by most of these isolates. Transcriptional repressor MecI proteolysis, which was shown to be dependent on MecR2 instead of MecR1 (ARÊDE & OLIVEIRA, 2013), is essential for the expression of β-lactam resistance. The MRSIG isolates that carried a SCC*mec* type II (2A) with an intact class A *mec* gene complex instead showed lower oxacillin MIC's values (< 0,5 - 2 μg/mL). The non-typable SCC*mec* elements had the same association of *ccr* genes (*ccrC* and *ccrA2B2*) as seen in SCC*mec* type II (2A), but lacked a *mec* gene complex structurally organized as in the classes A, B, C1 or C2. The wide variation in susceptibility to oxacillin (MIC's < 0,5 - 256 μg/mL) demonstrated by these isolates might be derived from new rearrangements of the *mecA*-regulatory genes. MRSIG isolates carrying a SCC*mec* type III (3A) or SCC*mec* composites exhibited high oxacillin resistance (MIC's ranging from 256 to ≥ 512 μg/mL).

Heterogeneous oxacillin resistance could be detected in an SCC*mec* composite-carrying MRSIG isolate from urine. The *mecA*-positive MRSIG isolate 36 exhibited an oxacillin MIC < 0,5 μg/mL by broth microdilution testing, but subpopulations of highly resistant cells (MIC ≥ 256 μg/mL) from the MRSIG 36 culture could be identified using a M.I.C. Evaluator strip (M.I.C.E., Thermo Fisher Scientific, Basingstoke, UK). Most clinical isolates of *mecA*-positive staphylococci express a heterogeneous oxacillin phenotype, in which most cells exhibit low-level oxacillin resistance while subpopulations are able to express higher oxacillin resistance levels. Hetero-resistant MRSIG isolates might be unrecognized by automated identification and susceptibility systems or conventional antimicrobial susceptibility testing in clinical laboratories, leading to β-lactam therapy failures. BD PHOENIX Automated Microbiology System predicted an oxacillin MIC ≥ 0.5 μg/mL for MRSIG 36, but it could not reliably determine susceptibility to methicillin in other five *mecA*-negative MRSIG isolates, which exhibited MIC < 0,5 μg/mL using the oxacillin broth microdilution test.

PBP2a latex agglutination testing yielded weak positive results for the oxacillin-heteroresistant isolate 36 and all MRSIG isolates with SCC*mec* type II (2A) or non-typable SCC*mec* elements, while stronger positive reactions were observed for MRSIG isolates with SCC*mec* type III (3A) or SCC*mec* composites. No correlation between the direct PBP2a test results and the oxacillin MIC values was observed.

Further investigation must be performed to characterize the SCC*mec* type variants found in these canine MRSIG isolates from various clinical infections, but PCR-based SCC*mec* typing could indicate the formation of genetic contexts enabling horizontal transfer of *mecA* that differ from those so far acknowledged as new for SIG by IWG-SCC. Methicillin resistance in staphylococcal species from companion animals raises concern, as β-lactams are veterinary critically important antimicrobials, and SIG isolates have the potential for zoonotic infections.

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DECLARTION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

RR performed the sample collection, bacterial isolation, antimicrobial susceptibility testing and PCR assays with collaboration of VTB, TLGFL, RASS, RJNC, SPV, DB and FZ. EMM and LMA planned the study and wrote the paper with collaboration of RTFM, VA, TGN, LAMG and CBD.

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