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Short Communication

Genetic analysis of *mec*A gene and detection of homologue *pbp*D in *Stahylococcus sciuri* group

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Abstract

Oxacillin/methicillin-resistance is related to the *mec*A and its regulatory genes *mecR1* and *mecI*. Its origin is still unknown, although evidences support that it is related to CNS, once *mec*A and a homologue gene, *pbpD*, were both detected in *Staphylococcus sciuri* species group. The present work evaluated 210 samples of skin and ear swabs from rodents and 60 nasal swabs from equines of Army Biologic Institute, Rio de Janeiro. Pheno- and genotypic characterization provided 59.52% (25/42) and 78.57% (11/14) *S. lentus* and *S. sciuri*, respectively. It was observed that although all *S. sciuri* isolates tested positive for *pbp*D, there was no correlation with oxacillin-resistance. On the other hand, isolates tested positive for *mec*A gene also presented phenotypic oxacillin-resistance in at least one assay. The alignment of the *mec*A gene showed that the nucleotide sequences were sorted into 2 different groups, one comprising the bovine strains and the other containing human and equine strains.

Key words: *Staphylococcus sciuri*, *mec*A, *pbp*D, antimicrobial resistance.

Antimicrobial resistance is a critical health issue around the world. Oxacillin/ methicillin-resistant *Staphylococcus aureus* represents an ever-increasing global threat especially considering vulnerable people (Souza *et al.*, 2012). Oxacillin/methicillin-resistance is related to the integration of a Staphylococcal cassette chromosome (SSC*mec*) in the bacterial chromosome made up of a *mec* gene complex, containing methicillin-resistance determinant *mecA* and its regulatory genes *mecR1* and *mecI*, and also the ccr gene complex, encoding site-specific recombinase responsible for the movement of the element. The *mec*A gene codifies a variant of penicillin-binding protein PBP2, termed PBP2a or PBP2', whose affinity with the beta-lactamic antibiotics is very low (Ito *et al.*, 2004). SCC *mec*has been identified not only in *S. aureus*but also in other coagulase-positive and coagulase-negative staphylococci. Its origin is still unknown, although evidences support that it is related to coagulase-negative staphylococci, once *mec*A and a homologue gene, *pbpD*, were both detected in *Staphylococcus sciuri* species group (Couto *et al.*, 2010, Tsubakishita *et al.*, 2010). S. sciuri species group comprises *S. sciuri*, *S. vitulinus*, *S. lentus*, and *S. fleurettii*. A recent report considered S. fleurettii and *S. sciuri* as the highly probable origin of the *mecA* gene (Tsubakishita *et al.*, 2010). The present work evaluated the oxacillin phenotypic antimicrobial resistance pattern and detected *mecA* and *pbpD* genes in S. sciurispecies group from rodents and equines of Army Biologic Institute, Rio de Janeiro. Also, *mec*A sequences from *sciuri* group strains were compared to others species in order to evaluate the degree of similarity among them.

A total of 210 samples from the skin and ear swabs of rodents and 60 from nasal swabs from the equines were analyzed and provided 56 S. sciurispecies group isolates. All 270 samples were aseptically collected and transported to the laboratory in coolers with ice (4-8 °C) for the bacteriological analyses. These samples were plated in duplicate in sheep blood agar (Oxoid), and incubated in aerobic conditions for 24 h at 37 °C. The colonies were identified according to routine microbiological diagnostics, including cul-

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tural properties, catalase and coagulase production. Then CNS isolates were submitted to phenotypic assays carried out as described by Stepanovic *et al.* (2005) considering the following tests: oxidase, novobiocin susceptibility, acid production from rafinose, mannose, arabinose, maltose, celobiose, galactose, lactose and salicin. All 56 samples phenotipically identified as Sciuri group were submitted to genotypic assays for *S. sciuri* and *S. lentus* were performed according to Yasuda *et al.* (2002) using the following primers: *16S rRNA Staphylococcus* spp. (756 bp) 5'- AAC TCT GTT ATT AGG GAA GAA CA - 3' and 5'- CCA CCT TCC TCC GGT TTG TCA CC - 3', *16S rRNA S. sciuri* (872 bp) 5'- GAA CCG CAT GGT TCA ATA G - 3' and 5'- GAC TCT ATC TCT AGA GCG G - 3', *16S rRNA S. lentus* (872 bp) 5'- GAA CCG CAT GGT TCA ATG T - 3' and 5'- AAC TCT ATC TCT AGA GCG A - 3'. Methicillin-resistance detection was performed according to the recommendations of the Clinical Laboratory Standard Institute (CLSI, 2012) through oxacillin and cefoxitin disk-diffusion test considering all strains presenting an inhibition zone diameter [#LWEQ#] 21 mm as resistant. Polymerase Chain Reactions (PCRs) for the detection of of *mec*A complex and *pbp*D genes was carried out as following: DNA was extracted according to the procedures described for the NucliSens® mini MAG System (BioMérieux). PCR for *mec*A gene (513 bp) was carried out using the primers outlined by Murakami *et al.* (1991) and methodology described by Coelho *et al.* (2007). PCRs for regulatory *mec*I and *mec*RI genes were carried out using the primers and methodology outlined by Rosato *et al.* PCR screening for *pbp*D gene (1020 bp) was carried out using the primers and methodology outlined by Couto *et al.* (2010): 5'- ATC CAT CAA TAT TGA ACC A -3' and 5'-TAT ATC TTC ACC AAC ACC -3'. Amplicons were detected by 1.5% agarose gel, stained with SYBR Green (Invitrogen) and examined under UV transilluminator (UvTrans). Also, three *mec*A gene sequences obtained from *S. sciuri* and *S. lentus* isolated from equines at the present study were included in a further step to evaluate the degree of similarity among *mec*A from *sciuri* group strains and others species. For this study, distinct primer pairs were designed to amplify different but overlapping segments of the whole *mec*A gene based on the nucleotide sequence of Staphylococcus *aureus* (HE681097) and *Staphylococcus sciuri* (AY820253) (Melo, 2013). The products of mecA gene PCR product were purified using Exo-Sap (USB Corporation) according to the manufacturer's recommended protocol. To assure fidelity, sequencing of both strands was performed at Helixxa Company, Brazil. Nucleotides sequences were edited using the software Bioedit version 7.0.9.0 (Hall, 1999) and assembled using the Mobyle@Pasteur program – (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::merger) (Rice *et al.*, 2000). Dendogram was performed by a neighbor joining (NJ) algorithm method using p-distance model with MEGA version 4.0 (Tamura *et al.*, 2007). The robustness of each branch was determined using the non-parametric bootstrap test (Felsenstein, 1985) with 1000 replicates.

Phenotypic identification provided 42 *S. lentus* and 14 *S. sciuri* isolates. After genotypic characterization, this profile had changed for 25 *S. lentus* (44.64%), being 24 from rodents and just one from equine, and 11 *S. sciuri* (19.64%), 8 from equine and 3 from rodents. Pheno- and genotypic results were concordant for 59.52% (25/42) and 78.57% (11/14) *S. lentus* and *S. sciuri* isolates, respectively. The remaining 20 isolates (35.72%) did not amplify the specific genes and from now on will be considered for this study as sciurispecies group. It is important to emphasize that this difference in species identification due to atypical biochemical patterns and that most routine diagnostic laboratories only perform phenotypic identification which can lead to misidentification. Genotypic characterization assays are more reliable, otherwise, it is still too expensive once equipments and skilled staff needs make it difficult to be used routinely. For such a long time, CNS *Staphylococcus* has been considered harmless and minor important species. Nowadays, the advances in molecular taxonomy and systematic allow researchers to a better comprehension of their role. Besides a direct clinical impact in diseases etiology, they are been investigated as reservoirs of resistance genes and consequently as part of the threat to the public represented by antimicrobial resistance. The *sciuri* group species are rarely associated to human or animal infections. Otherwise, similarities of *mec*A and *pbp*D genes suggest a closely related phylogeny (Couto *et al.*, 2010). In our study, *sciuri* species group were evaluated for oxacillin-resistance by means of oxacillin and cefoxitin disk diffusion and mecA and *pbp*D genes detection. It was detected a 14.29% oxacillin- and 8.93% cefoxitin-resistance pattern by disk diffusion assay. Stepanovic *et al.* (2005) detected oxacillin-resistant *S. sciuri* strains from human clinical samples. Bagcigil *et al.* (2007) isolated oxacillin-resistant CNSs from nasal cavities of equines exposed to antimicrobials, including β-lactams. PCRs for pbpD, *mec*A, *mec*I and *mec*RI genes revealed that 21.42% (12/56) isolates tested positive for *pbp*D, 10.71% (6/56) for mecA and 5.35% (3/56) for both *mec*I and *mec*RI. Although 12 isolates tested positive for *pbp*D, no correlation was observed with antimicrobial resistance for isolates that presented only *mec*A homologue (Table 1). These results agree to the reports of its relation to essential functions as growth and cell wall synthesis (Antignac and Tomasz, 2008). On the other hand, isolates tested positive for *mec*A gene also presented phenotypic resistance in at least one assay.

PCR using primers outlined by Melo (2013) based on the nucleotide sequence of S. aureus *mec*A gene (HE681097) successfully amplified *mec*A gene fragments from the two *S. sciuri* and one *S. lentus* from equine. The resulting *mec*A gene fragments were sequenced and the overlapping reads were assembled in contigs. Otherwise, newly

designed primers based on bovine S. *sciuri mec*A sequence (AY820253) tested negative for this isolates. Sequences of *mec*A gene from different hosts provided by our studies and available at NCBI GenBank were used to generate a dendrogram (Figure 1). Genomic divergences between *mec*A genes originated two different clusters of *Staphylo-*

Table 1 - Distribution of *sciuri* group *Staphylococcus* patterns.

Pattern (n*)	Disk diffusion		Genes				Species
	Oxacillin	Cefoxitin	*pbp*D	*mec*A	*mec*I	*mec*RI	
1 (2)	R**	R	+	+	+	+	*S. sciuri*
2 (1)	R	R	-	+	+	+	*S. lentus*
3 (1)	S*	S	+	-	-	-	*S. sciuri group*
4 (2)	R	R	-	+	-	-	*S. sciuri group*
5(1)	R	S	-	+	-	-	*S. sciuri group*
6(1)	R	S	-	-	-	-	*S. lentus*
7 (9)	S	S	+	-	-	-	*S. sciuri*

*n: number of isolates; ** R: resistant; S: sensible.

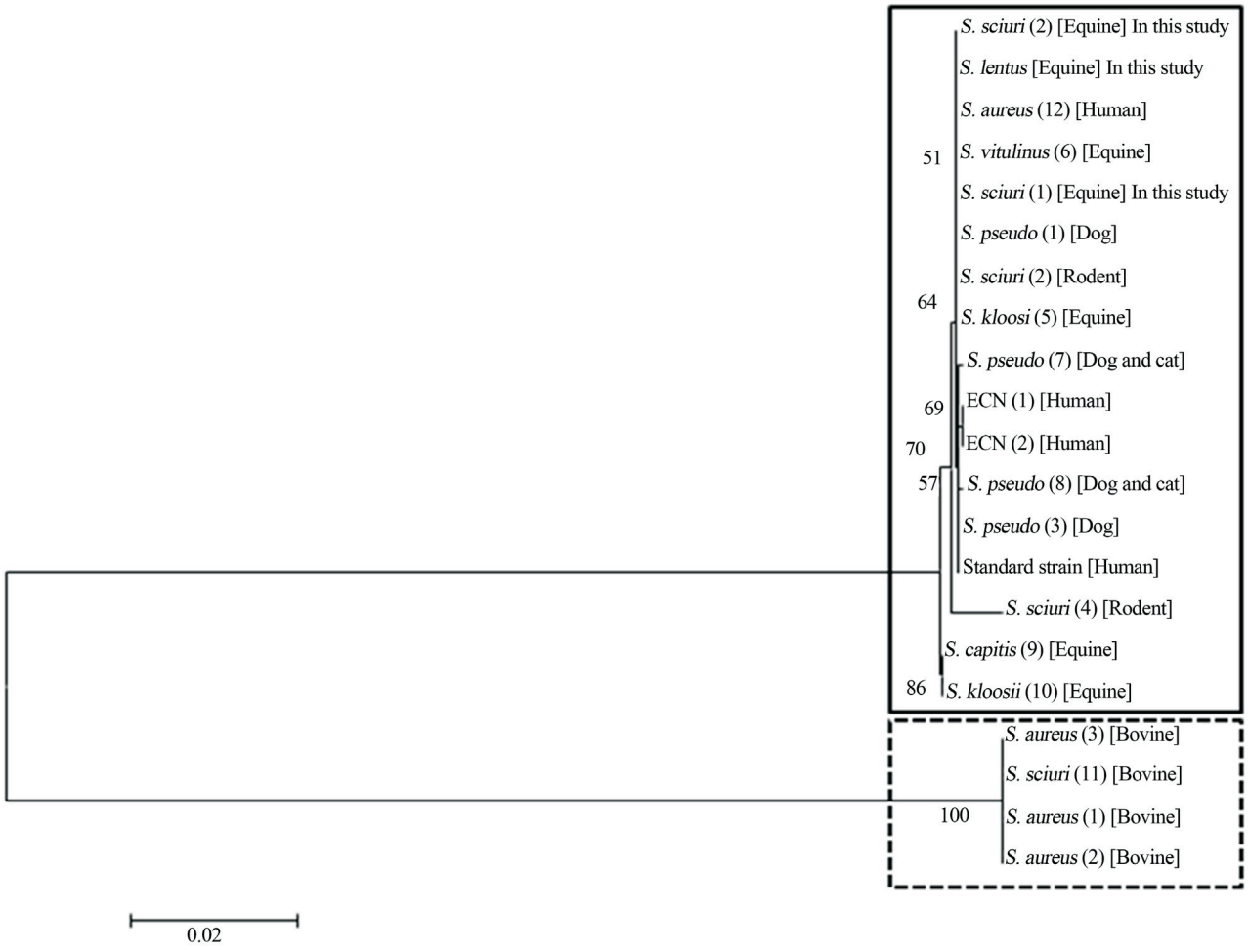

Figure 1 - Dendrogram showing the genetic divergence of nucleotide sequences of bovine (dotted line) and others species (continuous line) Staphylococcal *mec*A gene. Sequences available of GenBank: S. pseudo (1): *S. pseudintermedius:* AM904731; S. sciuri (2): *S. sciuri:* Y13096; S. pseudo (3): *S. pseudintermedius:* AM904732; S. sciuri (4): *S. sciuri:* Y13095; S. kloosii (5): *S. kloosii:* AM048803; S. vitulinus (6): *S. vitulinus:* AM048802; S. pseudo (7): *S. pseudintermedius:* EU929082; S. pseudo (8): S .*pseudintermedius:* EU929081; S. capitis (9): *S. capitis:* AM048805; S. kloosii (10): *S. kloosii:* AM048804; S. sciuri (11): *S. sciuri:* AY820253; S. aureus (12): *S. aureus:* HE681097. Sequences of Melo (10): Standard Strain: *S. aureus;* ECN (1) and (2): CNS isolates 1 and 2; S. aureus (1), (2) and (3): *S. aureus* isolates 1, 2 and 3. Sequences of this study: S.sciuri (1) and (2): *S. sciuri* isolates 1 and 2*;* S. lentus: *S. lentus.*

coccus spp., one comprising dog, cat, rodent, equine and human isolates and the other just bovine isolates (Figure 1). The puzzling question concerning *mec*A gene evolutive pathway is being investigated for researches around the world. The idea that it has been generated in or transferred into a broad-host-range *Staphylococcus* species is well established. Also it is recognized that the *mecA* region carried by SCC *mec* might have spread beyond the host animal species (Coelho *et al.*, 2007). Otherwise, there are some blanks to be filled such as SCC *mec*is generated in*S. aureus,* which is both a human- and animal-related*Staphylococcus*species.

We have been recently confronted with the rapid spread of community-acquired MRSA and facilitated transmission of animal-derived MRSA to humans (Bagcigil *et al.*, 2007; Coelho *et al.*, 2007; Furowicz, 2010). In the present study, *mec*A from equine *S. sciuri* and *S. lentus* was similar to *mec*A from human *S. aureus* whereas divergent to *mec*A from bovine *S. sciuri*. These findings outline the hypothesis that the evolution of a resistance gene is more closely related to hosts environment, that bacterial specie. Tenover (2008) in his article "Vancomicin-resistant *Staphylococcus aureus*: a Perfect but Geographically Limited Storm?" gives us a clue that antibitioc resistance issue is not so simple answer. Science is a creative activity that request exploration and gambling. We have to be open-minded to understand that some evolutionary steps are more successful than others and the pathways to resistance are not so predictable. The biggest challenge is keep researching in order to enhance our knowledge of the mechanisms beyond resistance, the evolutionary pathways of resistance among microorganisms, and selective pressure factors that contribute to the expression of underlying genes (Souza *et al.*, 2012).

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