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Research Paper

Impairments of *mec*A gene detection in bovine *Staphylococcus* spp.

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Abstract

Staphylococcus aureus antimicrobial resistance, especially to beta-lactams, favors treatment failures and its persistence in herd environment. This work aimed to develop a more specific primer for *mec*A gene detection based on the comparison of the conserved regions from distinct host origins and also investigated the presence of homologue *mec*A_{LGA251} in bovine strains. A total of 43 *Staphylococcus* spp. were included in this study, comprising 38 bovine *S. aureus*, two human and three equine coagulase-negative staphylococci (CNS). Phenotypical methicillin-resistance detection was performed through oxacillin agar-screening and cefoxitin disk-diffusion test. None isolate tested positive for *mec*A_{LGA251} gene. For *mec*A gene PCR, new primers were designed based on the sequences of human *S. aureus* (HE681097) and bovine *S. sciuri* (AY820253) *mec*A. The new primers based on the *S. aureus mec*A sequence amplified fragments of human and equine CNS and the ones based on *S. sciuri mec*A sequence only yielded fragments for *S. aureus* bovine strains. Multiples alignments of *mec*A gene sequences from bovine, human and equine revealed punctual but significant differences in bovine strains that can lead to the *mec*A gene detection impairment. The observed divergences of *mec*A gene sequences are not a matter of animal or human origin, it is a specificity of bovine samples.

Key words: antimicrobial resistance, bovine mastitis, *Staphylococcus*, *mec*A gene.

Introduction

Staphylococcus spp. is the most commonly isolated agent in bovine mastitis, especially considering *Staphylococcus aureus* (Coelho *et al.*, 2009). Its importance is not only because of its distribution and pathogenicity but also due to its ability to overcome antimicrobial effects. Its high antimicrobial resistance level, especially to beta-lactams, favors treatment failures and its persistence in herd environment. Bacterial resistance mechanisms to this antimicrobial class include production of ß-lactamases and low-affinity penicillin-binding protein 2a (PBP2a) determined by the presence of the chromosomal genes *bla* and *mec*A, respectively. The latter, involved in oxacillin-resistance, precludes human therapy with any of the currently available ß-lactam antibiotics, and is considered to predict resistance to several classes of antibiotics (Moon *et al.*, 2007). Once phenotypic expression of beta-lactam resistance in *Staphylococcus* isolates is usually heterogeneous, the amplification of *mec*A gene is prescribed as a gold standard in the detection of this resistance (CLSI, 2012).

Otherwise, our research results in animal science field concerning to bovine staphylococci beta-lactam resistance does not support that *mec*A gene plays the same significant role in the detection of this resistance. As a matter of fact, our previous studies reported several phenotypically oxacillin-resistant isolates that tested negative for *mec*A gene (Coelho *et al.*, 2009; Mendonça *et al.*, 2012; Soares *et al.*, 2012).

Theories have been proposed to explain the lack of correlation between the observed phenotypical methicillin-resistance and the detection of *mec*A gene. The occurrence of deletions, insertions or point mutations could have altered the original nucleotidic sequence in *mec*A gene

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primer alignment region in bovine isolates, as reported (Sakoulas *et al.*, 2001). Also, a recently published article (García-Álvarez *et al.*, 2011) reported the presence of MRSA strains with unusual features in bovine milk samples from the UK. These strains carried a novel *mec*A gene that was only 70% identical at the nucleotide level to the classical *mec*A gene, escaping detection by routine PCR assays. The novel *mec*A was named *mec*A_{LGA251}. The gene *mec*A_{LGA251} is part of a novel SCC*mec* element, identified as type XI. Human clinical isolates with *mec*A_{LGA251} were reported in the UK and Denmark. The ability of these strains to cause infections in humans was confirmed by a concurrent report describing two patients infected with similar strains in Ireland (Shore *et al.*, 2011).

Considering these hypothesis and that the true scope of MRSA in animals and its impact on human health are still only superficially understood we decided to investigate the presence of *mec*A_{LGA251} in the bovine isolates tested negative for *mec*A. Furthermore, *mec*A sequences from human and animal origins stored in NCBI databases were analyzed in order to develop a more specific primer considering distinct conserved regions in the gene.

Materials and Methods

Bacterial strains

A total of 43 previously identified isolates were included in this study. Thirty eight *Staphylococcus aureus* from milk and two Coagulase-Negative *Staphylococcus* (CNS) from milker's hands and noses from dairy cattle farm properties at the state of Rio de Janeiro, Brazil. Also, three CNS strains, two *S. sciuri* and one *S. lentus*, collected from horses in the Army Biology Institute, Rio de Janeiro provided by Silva *et al.* (2013, unpublished results). A human *S. aureus* standard strain (ATCC43300) was used as control.

Phenotypic detection of methicillin-resistance

Methicillin-resistance detection in *Staphylococcus* strains was performed according to the recommendations of the Clinical Laboratory Standard Institute (CLSI, 2008; CLSI, 2012). The agar-screening test was performed on Mueller-Hinton agar (Himedia) supplemented with 4% (w/v) NaCl containing oxacillin at 6 μg/mL where bacterial growth 24 h at 35 Â°C indicates oxacillin- resistance. Also, strains were screened by cefoxitin disk-diffusion test considering an inhibition zone diameter ≤ 21 mm as resistant. *S. aureus* standard strains ATCC43300 and ATCC29213 were used as control.

Detection of *mec*A and *mec*A_{LGA251} gene by PCR

A 1.5 mL overnight culture of a single *Staphylococcus* colony was centrifuged (three times) and the cell pellet was suspended in 600 μL of lysis solution (200 mM TrisHCl, 25 mM EDTA, 25 mM NaCl, 1% SDS, pH8.0) at 65 °C for 30 min. The DNA was extracted with Chloroform:Isoamyl Alcohol 25:24:1 twice and precipitated by ice-cold ethanol two volumes. DNA pellet was washed with 70% ethanol and resuspended in 30 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0) and stored at -20 °C until used. PCR for *mec*A gene was initially carried out using the primers and methodology outlined by Murakami *et al.* (1991): 5'-AAA ATC GAT GGT AAA GGT TGG C-3' and 5'-AGT TCT GCA GTA CCG GAT TTG C-3'. PCR screening for *mec*A_{LGA251} gene was carried out using the primers and methodology outlined by Cuny *et al.* (2011): 5'-GCT CCT AAT GCT AAT GCA-3' and 5'-TAG CAA TAA TGA CTA CC-3'. Amplicons were detected by 1.5% agarose gel, stained with SYBR Green (Invitrogen) and examined under UV transilluminator (UvTrans).

Design of primers based on GenBank database sequences of *mec*A gene from *Staphylococcus* genus

Firstly, a range of nucleotide sequences of *Staphylococcus* spp. *mec*A gene was obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/) and aligned using ClustalW (Higgins *et al.*, 1994) within the MEGA version 4.0 program (Tamura *et al.*, 2007). From the resultant alignment, a representative sequence of human S. *aureus* *mec*A (HE681097) was used to design the primers using the Primer3 website (http://frodo.wi.mit.edu/) (Rozen and Skaletsky 2000). Four distinct primer pairs were designed to amplify different but overlapping segments of the whole *mec*A gene. Table 1 displays the primer sequences considering the four covered regions: anterior (mecAant), internal 2 (mecAint2), internal 1 (mecAint1) and posterior (mecApos). The unique primer that tested positive for both human and bovine isolates was the internal 1 (mecAint1). The alignment of its sequenced amplicon yielded a maximal level of identity with the bovine *S. sciuri* mecA (AY820253) on BLASTn. In a further step, a new set of three distinct primers was designed considering the following regions: anterior (mecSsciuriAnt), internal (mecSsciuriInt), and posterior (mecSsciuriPos). PCR assays were carried out using the newly designed primers plus the previous designed mecAint1 in order to cover the whole *mec*A gene (Table1). Figure 1 displays the schemes of the highly conserved regions used to design *mec*A primers for *S. aureus* (1A) and *S. sciuri* (1B).

Sequencing and sequence analysis

PCR products 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.

Nucleotide sequences were edited using the software Bioedit version 7.0.9.0 (Pichon *et al.*, 2011) and compared to sequences deposited in GenBank using the Blastn algo-

Table 1 - Sequences of the primer sets designed in this study for the amplification of *mec*A gene.

Primer	Sequence (5' - 3')	PCR product	Pro-gram*
mecAantF mecAantR	CAT ATC GTG AGC AAT GAA CTG A GGC CAA TTC CAC ATT GTT TC 641 bp 1		
mecAint2F mecAint2R	TCC AGG AAT GCA GAA AGA CC TCA CCT GTT TGA GGG TGG A 669 bp	1	
mecAint1F mecAint1R	GGC TAT CGT GTC ACA ATC GTT TCA CCT TGT CCG TAA CCT GA	689 bp	1
mecAposF mecAposR	GCA CTC GAA TTA GGC AGT AAG AA AGC AAC CAT CGT TAC GGA TT	631 bp	1
mecSciuriAntF mecSsciuriAntR	AGC CAT CGT GGT TGT AAT CAT CAA TGC CAA CTT CAT GTG CT 500 bp 2		
mecSsciuriIntF mecSsciuriIntR	CAG GCA TGC AGA AAA ATC AA TTG AGT CGA ACC AGG TGA TG 809 bp 2		
mecSsciuriPosF mecSsciuriPosR	AGG TTA TGG ACA AGG CGA AA AAA TCG TCA TAC ACT TTT CCA GA 417 bp 2		

*1. 5 min at 94 °C, 30 x (1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C), and 10 min at 72 °C, 2. 5 min at 94 °C, 30 x (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) and 10 min at 72 °C.

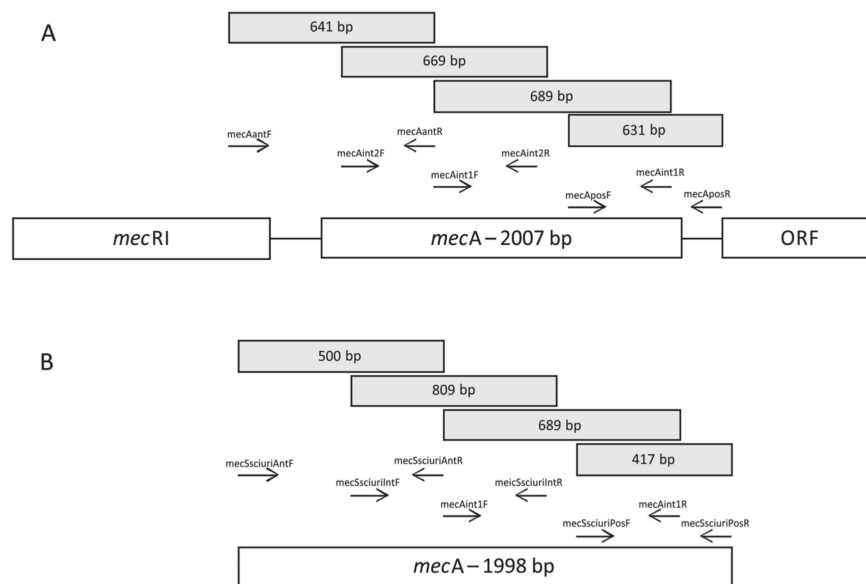

Figure 1 - Schematic diagram of *mec*A gene of *Staphylococcus* aureus (HE681097) (A) and *Staphylococcus sciuri* (AY820253) (B) displaying the location of designed primers.

rithm (Altschul *et al.*, 1997). The sequences were imported into the program MEGA version 4.0, and aligned in Clustal W program. The resulting multiple alignments were optimized visually. The final alignment of this work is available on request. The sequence contigs of *mec*A gene were assembled using the Mobyle@Pasteur program - (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::merger) (Rice *et al.*, 2000). The sequences were deposited in a GenBank database and compared with other sequences (Table 2). Dendogram was performed by a neighbor joining (NJ) algorithm method using p-distance model with MEGA (Tamura *et al.*, 2007). The robustness of each branch was determined using the non-parametric bootstrap test (Felsenstein 1985) with 1000 replicates.

Results

From thirty-eight bovine *S. aureus* isolates, twenty were found oxacillin-resistant at the phenotypical assays, but none tested positive for *mec*A gene using primers set specified by Murakami *et al.* (1991). Also, bovine *mec*A-negative isolates also tested negative for $mecA_{LGA251}$ gene described by Cuny *et al.* (2011). Otherwise, the oxacillin-resistant CNS isolates of human origin tested positive for *mec*A gene (Table 3).

All 43 *Staphylococcu*s isolates were tested for *mec*A gene by PCR using the primers set (mecAant, mecAint2, mecAint1, and mecApos) based on the human *S.aurues mec*A sequence (HE681097). These primers successfully amplified *mec*A gene fragments from the two CNS and the standard strain from human and for the two *S. sciuri* and one *S. lentus* from equine whereas only three bovine *Staphylococcus* strains were positive with only one (mecAint1) of the four primers set (Table 4).

Fragments originated from the mecAInt1 primer set amplification were sequenced and aligned in order to evaluate the differences in the nucleotide sequences. It was

observed punctual differences in the alignment when comparing the bovine to the human and equine strains. Also it was observed differences in the primer annealing site of the established forward primer specified by Murakami *et al.* (1991) (Figure 2). The comparison of the sequences from our bovine strains with others sequences available in NCBI databases provided a fully identity (100%) to the *mec*A gene from bovine *S. sciuri* (AY820253).

Table 2 - GenBank accession numbers for *mec*A sequences of *Staphylococcus* spp. isolates and respective hosts used for dendogram assembly.

Species	Host	GenBank accession number
S. pseudintermedius	Dog	AM904731
S. sciuri	Rodent	Y13096
S. pseudintermedius	Dog	AM904732
S. sciuri	Rodent	Y13095
S. kloosii	Equine	AM048803
S. vitulinus	Equine	AM048802
S. pseudintermedius	Dog and Cat	EU929082
S. pseudintermedius	Dog and Cat	EU929081
S. capitis	Equine	AM048805
S. kloosii	Equine	AM048804
S. sciuri	Bovine	AY820253
S. aureus	Human	HE681097
*S. aureus**	Human	KF058908
CNS*	Human	KF058904
CNS*	Human	KF058903
*S. aureus**	Bovine	KF058901
*S. aureus**	Bovine	KF058902
*S. aureus**	Bovine	KF058900
*S. sciuri**	Equine	KF058905
*S. sciuri**	Equine	KF058906
*S. lentus**	Equine	KF058907

*Obtained from this study.

Table 3 - Distribution of phenotypical beta-lactam resistance pattern with the corresponding detection of *mec*A gene and homologue *mec*A_{LGA251}.

Pattern/ isolates (n)	OAS	CDD	mecA gene*	mecA$_{LGA251}$†
1(7)	R	R	-	-
2(2)	R	S	-	-
3(11)	S	R	-	-
4(18)	S	S	-	-
5 (1 CNS)	R	R	+	-
6 (1 CNS)	S	R	+	-

R - resistant; S - sensible; OAS - oxacillin agar-screening test; CDD - cefoxitin disk-diffusion test; (-) negative; (+) positive.
*Murakami *et al.* (1991); †Cuny *et al.* (2011).

Table 4 - Staphylococcal *mecA* gene detected for human, equine and bovine isolates.

Origin	Isolates	mecA*	mecA ant	mecA int2	mecA int1	mecA pos	mec Ssciuri Ant	mec Ssciuri Int	mec Ssciuri Pos
Milk (Bovine)	*S. aureus* (1)	-	-	-	+	-	+	+	+
Milk (Bovine)	*S. aureus* (2)	-	-	-	+	-	+	+	+
Milk (Bovine)	*S. aureus* (3)	-	-	-	+	-	+	+	+
Hands (Human)	ECN (1)	+	+	+	+	+	-	-	-
Nasal (Human)	ECN (2)	+	+	+	+	+	-	-	-
Standard Strain (Human)	*S. aureus*	+	+	+	+	+	-	-	-
Nasal (Equine)	*S. sciuri* (1)	+	+	+	+	+	-	-	-
Nasal (Equine)	*S. sciuri* (2)	+	+	+	+	+	-	-	-
Nasal (Equine)	*S. lentus*	+	+	+	+	+	-	-	-

*Murakami *et al.* (1991).

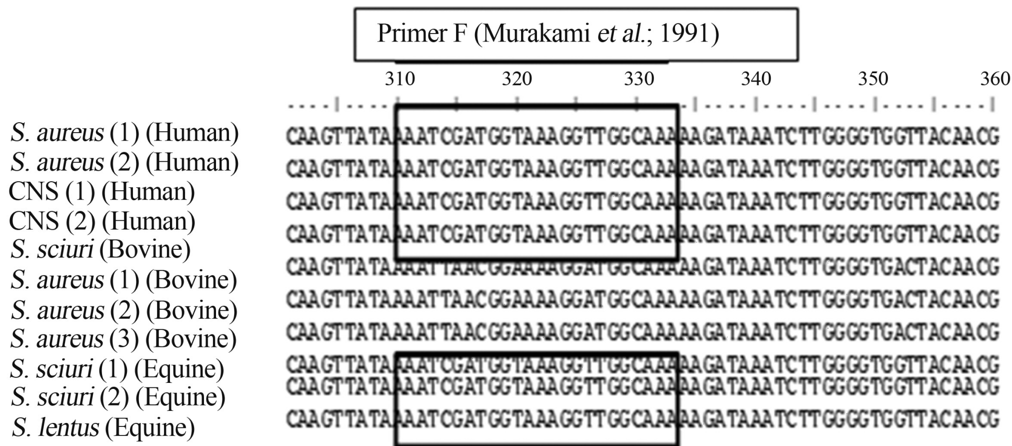

Figure 2 - Multiple alignments of sequences of *mec*A gene fragments generated by amplification using the first-step primers set mecAint1. Analyzes of the sequences segments of *mec*A genes from different hosts (bovine, human and equine) revealed that the ones from bovine origin present punctual differences. The highlighted box displays the primer F's annealing site based on human *S. aureus mec*A gene specified by Murakami *et al.* (1991) detaching the punctual nucleotide differences that possibly impaired the annealing and amplification of *mec*A gene from our bovine strains. *S. aureus* (1) (Human): (HE681097); *S. aureus* (2) (Human): standard strain (ATCC43300) (KF058908); CNS (1) and (2): CNS isolates 1 and 2 (human origin) (KF058904, KF058903, respectivaly); S. *aureus* (1, 2 and 3): *S. aureus* isolates 1, 2 and 3 (bovine origin) (KF058901, KF058902, KF058900, respectivaly); *S. sciuri* (1and 2): *S.sciuri* isolates 1 and 2 (equine origin) (KF058905, KF058906, respectivaly) and *S. lentus*: *S. lentus* isolate (equine origin) (KF058907, respectivaly).

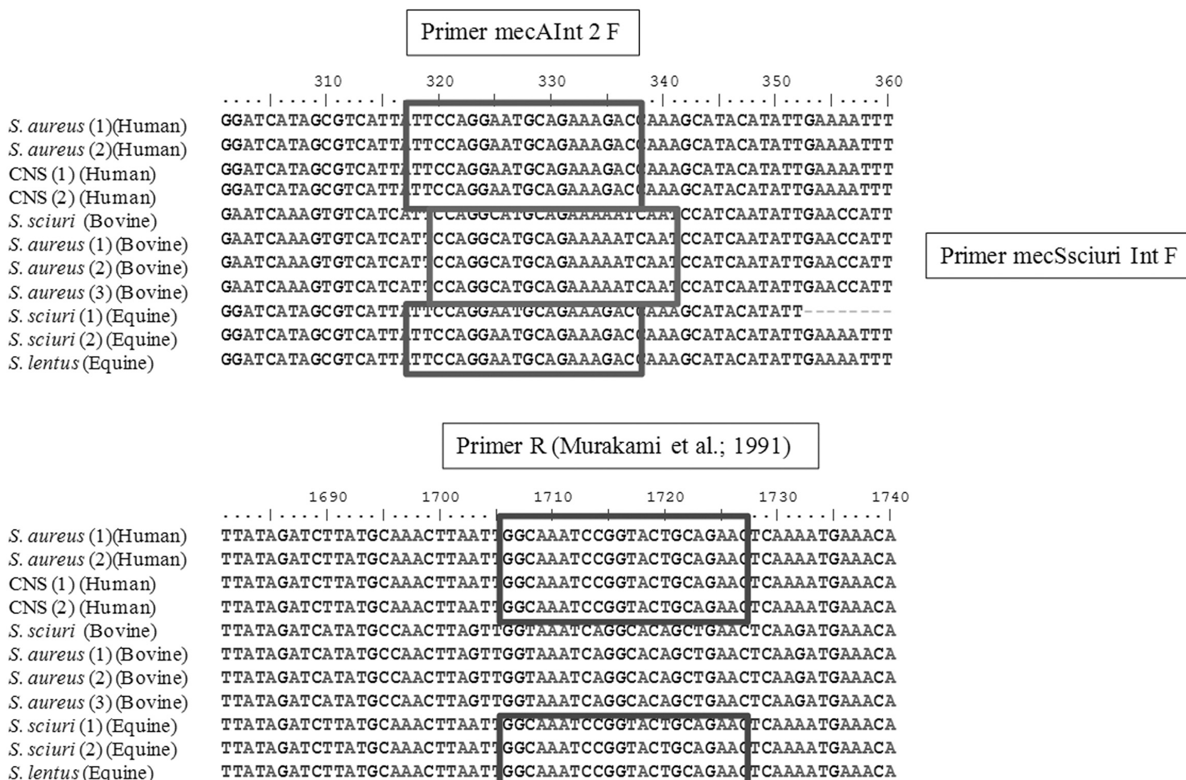

Figure 3 - Multiple alignments of contigs of *mec*A gene sequences generated by amplification using both the first (*mec*Aant, mecAint1, *mec*Aint2, *mec*Apos) and second-step (*mec*SsciuriAnt, *mec*SsciuriInt, *mec*SsciuriPos) primers set. Analyzes of the *mec*A genes sequences of different hosts (bovine, human and equine) revealed that the ones from bovine origin present punctual differences. The highlighted boxes display the primer annealing sites(Primer mecAint2 F, Primer mecSsciuriInt F and Primer R from Murakami *et al.* (1991)). The *in silico* analysis of these regions detaches punctual nucleotide differences that can lead to the *mec*A gene detection impairment in bovine strains. *S. aureus* (1) (Human): (HE681097); *S. aureus* (2) (Human): standard strain (ATCC43300) (KF058908); CNS (1) and (2): CNS isolates 1 and 2 (human origin) (KF058904, KF058903, respectivaly); S. *aureus* (1, 2 and 3): *S. aureus* isolates 1, 2 and 3 (bovine origin) (KF058901, KF058902, KF058900, respectivaly); *S. sciuri* (1and 2): *S.sciuri* isolates 1 and 2 (equine origin) (KF058905, KF058906, respectivaly) and *S. lentus*: *S. lentus* isolate (equine origin) (KF058907).

All eight isolates comprising two CNS and one standard strain from human, three CNS from equine, and three *S. aureus* bovine strain that tested positive for the *mec*A gene amplification carried out with at least one of the first-step designed primers set (mecAant, mecAint2, mecAint1, and mecApos) were submitted to a PCR using the primers designed at the second-step (mecSsciuriAnt, mecSsciuriInt and mecSsciuriPos) based on *S. sciuri* sequence of *mec*A gene (AY820253) (Table 1, Figure 1B). In such a different way, the result of this PCR assay showed that only the three bovine strains amplified *mec*A gene segments (Table 4). The resulting bovine, human and equine *mec*A gene fragments were sequenced and the overlapping reads were assembled in contigs.

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 (Figure 3). Punctual mutations in the primer annealing sites to detect *mec*A described by Murakami *et al.* (1991) explain why only the human and equine *mec*A gene were amplified and the fail to detect this gene from bovine isolates (Table 4, Figure 3). Moreover, all primer annealing sites for *mec*A gene available in the literature were evaluated and do not aligned in none of the *mec*A sequences obtained from the bovine isolates used in this study (Oliveira and Lencastre, 2002; Tan, 2002; Zhang *et al.*, 2004; Baddour and Abuelkeir, 2007). Also the in silico analysis of the primer annealing sites of the newly designed primers set used in this study confirm PCR results shown in Table 4.

Sequences of *mec*A gene of *Staphylococcus* spp. from different hosts provided by our study and available at NCBI GenBank were used to generate a dendogram (Table 2, Figure 4). Genomic divergences between *mec*A genes originated two different clusters of *Staphylococcus* spp., one comprising dog, cat, rodent, equine and human isolates and the other just bovine isolates.

Discussion

Livestock associated *Staphylococcus* spp. seems to be an additional challenge for veterinary research. After a decade of dairy science research concerning antimicrobial resistance we were really puzzled with the low correlation observed between phenotypic assays for detection of beta-lactam resistance and *mec*A gene detection.

Once literature reported the existence of a homologue gene, *mec*A$_{LGA251}$, with unusual features in bovine milk samples that was not possibly detected by routine PCR assays (Cuny *et al.*, 2011; García-Álvarez *et al.*, 2011), all

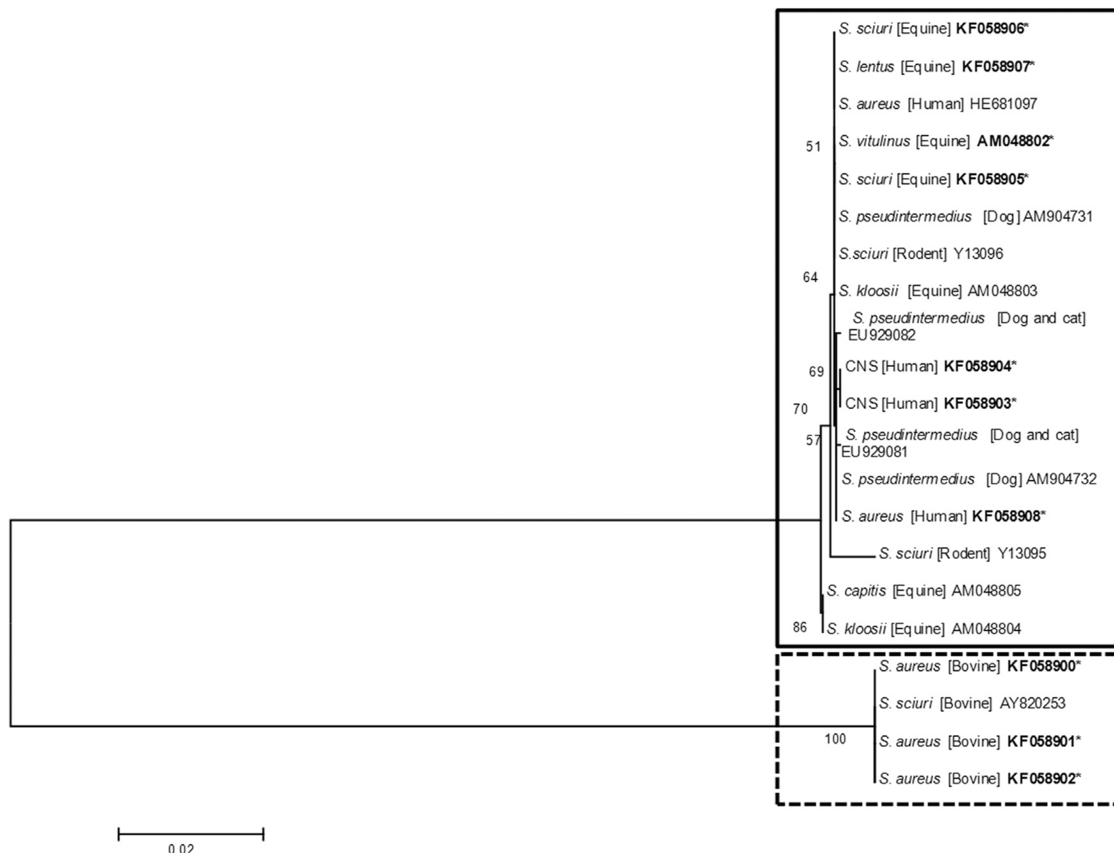

Figure 4 - Dendogram showing the genetic divergence of nucleotide sequences of bovine (dotted line) and others species (continuous line) Staphylococcal *mec*A gene. *Obtained from this study.

phenotypic oxacillin-resistant strains of this study were tested for this gene, no one was positive.

Assuming that all steps of PCR techniques were well-established and its efficiency and reliability were not in question, a critical point was left for us to investigate: the selection of the primers for *mec*A gene detection.

Recently, literature reports that the majority of *Staphylococcus aureus* isolates that are recovered from humans or cattle represent genetically distinct sets of clonal groups with evidence of host specialization among them (Herron-Olson *et al.*, 2007; Sakwinsk *et al.*, 2011).

This statement supports the idea that the use of human parameters in the analysis of beta-lactam resistance considering detection of *mec*A gene could lead to the misidentification of this resistance. This idea was supported by our results that the primer F's annealing site based on human *S. aureus mec*A gene specified by Murakami *et al.* (1991) presented punctual nucleotide differences that possibly impaired the annealing and amplification of *mec*A gene from our bovine strains.

The two-step procedure for primers design took in account sequences of distinct conserved regions in the *mec*A gene. In the first step, the newly synthesized primers were based on the nucleotide sequences of *mec*A gene of *S. aureus* (HE681097). Those primers were successful in amplifying *mec*A gene segment of human and equine *Staphylococcus* but mostly failed in bovine strains that tested positive only for the first internal segment primers set (mecAint1). Otherwise, the second-step primers set based on sequence of *S. sciuri mec*A gene (AY820253) only yielded *mec*A gene segments for bovine strains. Analyzes of the multiple alignments of the contigs of *mec*A gene sequences from bovine, human and equine origins revealed that the ones from bovine origin presented punctual differences. As far as we can see, these differences in the nucleotide sequences are significant, detaching that the primer annealing sites described in literature (Murakami *et al.*, 1991; Oliveira and Lencastre, 2002; Tan, 2002, Zhang *et al.*, 2004, Baddour and Abuelkeir, 2007) were not able in the *in silico* analysis to anneal to the bovine sequence what can lead to the *mec*A gene detection impairment in bovine strains.

The impairment of the *mec*A gene detection in bovine isolates is related to the punctual but significant differences in primer annealing sites once mostly primers set are usually based on human *mec*A gene sequences. This impairment was not noticed for equine isolates since that the divergence of *mec*A gene sequences are not a matter of animal or human origin, it is a specificity of bovine samples.

Acknowledgments

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