Short communication: β -Lactam resistance and vancomycin heteroresistance in *Staphylococcus* spp. isolated from bovine subclinical mastitis

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ABSTRACT

The use of antimicrobial agents has led to the emergence of resistant bacterial strains over a relatively short period. Furthermore, *Staphylococcus* spp. can produce β -lactamase, which explains the survival of these strains in a focus of infection despite the use of a β -lactam antibiotic. The aim of this study was to evaluate the resistance of *Staphylococcus* spp. isolated from bovine subclinical mastitis to oxacillin and vancomycin (by minimum inhibitory concentration) and to detect vancomycin heteroresistance by a screening method. We also evaluated β -lactamase production and resistance due to hyperproduction of this enzyme and investigated the *mecA* and *mecC* genes and performed staphylococcal cassette chromosome *mec* typing. For this purpose, 181 *Staphylococcus* spp. isolated from mastitis subclinical bovine were analyzed. Using the phenotypic method, 33 (18.2%) of *Staphylococcus* spp. were resistant to oxacillin. In contrast, all isolates were susceptible to vancomycin, and heteroresistance was detected by the screening method in 13 isolates. Production of β -lactamase was observed in 174 (96%) of the *Staphylococcus* spp. isolates. The *mecA* gene was detected in 8 isolates, all of them belonging to the species *Staphylococcus epidermidis*, and staphylococcal cassette chromosome *mec* typing revealed the presence of type I and type IV isolates.

Key words: β -lactam, resistance, *mecC* and *mecA*, staphylococci, cattle

Short Communication

As one of Brazil's most important agricultural products, milk and milk products play a relevant economic

and social role, generating income and employment for the population (Carvalho et al., 2003). The indiscriminant use of antibiotics, including vancomycin, one of the last therapeutic options for staphylococcal infections, has selected for strains with reduced susceptibility to this drug (Schwalbe et al., 1987). According to Natoli et al. (2009), the heteroresistant phenotype of these strains may be associated with treatment failure or may be a precursor of glycopeptide resistance.

The objective of the present study was to evaluate resistance of *Staphylococcus* spp. isolated from bovine subclinical mastitis to oxacillin and vancomycin by a phenotypic method (MIC) and to detect vancomycin resistance by a screening method. Additionally, *Staphylococcus* spp. isolates were submitted to determination of β -lactamase production and hyperproduction and investigation of the oxacillin resistance *mecA* and *mecC* genes. Staphylococcal cassette chromosome *mec* (*SCCmec*) typing was performed on isolates carrying the resistance gene.

A total of 181 *Staphylococcus* spp. strains isolated from bovine subclinical mastitis cases in 6 Brazilian states, maintained in the collection of Embrapa Dairy Cattle (Juiz de Fora, Brazil), were studied. Bacterial isolation and identification were performed according to the National Mastitis Council (NMC, 2004) using coagulase and sugar (trehalose, maltose, and mannitol) fermentation tests. Strains belonging to the CNS group were submitted to biochemical tests as proposed by Cunha et al. (2004) for phenotypic identification of the species. Total DNA was extracted using the Illustra kit (GE Healthcare, Little Chalfont, UK). Genotypic identification of CNS was performed using primers targeting conserved sequences adjacent to the 16S and 23S genes by the internal transcribed spacer-polymerase chain reaction (ITS-PCR) described by Couto et al. (2001), using primers G1 and L1. Polymerase chain reaction using the Staur-4 and Staur-6 primers developed by Straub et al. (1999) was used for *S. aureus*. Amplifica-

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tion efficiency was monitored by 2% agarose gel electrophoresis stained with Saber Safe DNA Gel Strain (São Paulo, Brazil) and viewed under a UV transilluminator.

The in vitro susceptibility of the isolates to oxacillin and vancomycin was tested by determining the MIC of these drugs using E-test (AB Biodisk, Solna, Sweden) as follows: Mueller-Hinton culture medium, inoculum size matching the turbidity of a 0.5 McFarland standard, incubation at 35°C, and MIC determination as the value corresponding to the intersection of the ellipse of bacterial growth inhibition. The interpretation followed the breakpoints proposed by CLSI (2015), which suggests oxacillin breakpoints for *S. aureus* as follows: susceptibility ≤ 2 $\mu\text{g/mL}$; resistance ≥ 4 $\mu\text{g/mL}$; and for CNS: susceptibility ≤ 0.25 $\mu\text{g/mL}$; resistance ≥ 0.5 $\mu\text{g/mL}$. The vancomycin breakpoints for *Staphylococcus* spp. were susceptibility < 4 $\mu\text{g/mL}$; resistance ≥ 4 $\mu\text{g/mL}$.

The PCR for the detection of *mecA* was performed using primers *mecA1* (AAAATCGATGGTA-AAGGTTGG) and *mecA2* (AGTTCTGCAGTACCG-GATTTG) according to the parameters described by Murakami et al. (1991). International reference strains were included in all reactions: positive control (*Staphylococcus aureus* ATCC 33591) and negative control (*Staphylococcus aureus* ATCC 25923). The presence of the *mecC* gene was investigated in isolates in which the *mecA* gene was not detected. The primers and parameters described by García-Álvarez et al. (2011) were used. Typing of SCC*mec* was performed using the method of Machado et al. (2007), which characterizes types I, II, III, and IV isolates, considering the corresponding molecular weights. As a control for the SCC*mec* typing, COL strains for SCC*mec* type I were used; N315 for SCC*mec* type IA; PER34 for SCC*mec* type II; AN546 for SCC*mec* type III; HU25 for SCC*mec* type IIIA and MW2 for SCC*mec* type IV. The reaction parameters were 92°C for 3 min followed by 30 cycles of 92°C for 1 min, 56°C for 1 min, and 72°C for 1 min and 30 s.

Production of β -lactamase was detected using nitrocefin-impregnated disks placed on *Staphylococcus* spp. colonies previously incubated at 35°C for 24 h on a Mueller-Hinton agar plate with the E-test oxacillin strip. The positive reaction was evidenced by the development of a red color and the negative color change. International reference strains were used as positive (*Staphylococcus aureus* ATCC 33591) and negative (*Staphylococcus xylosum* ATCC 29979) controls.

Isolates that tested negative for the *mecA* gene and exhibited oxacillin resistance by the phenotypic method were analyzed to determine whether they were hyperproducers of β -lactamase using a disk containing amoxicillin (20 μg) and clavulanic acid (10 μg).

The susceptibility breakpoint was the formation of an inhibition halo of 20 mm after incubation for 24 h at 35°C (Ghoshal et al., 2004). The screening medium was prepared with brain heart infusion agar (BHI) containing 4 $\mu\text{g/mL}$ (Hiramatsu, 2001) or 6 $\mu\text{g/mL}$ vancomycin (CLSI 2015). *Staphylococcus aureus* (ATCC 29213) susceptible to vancomycin and vancomycin-resistant *Enterococcus faecalis* (ATCC 51299) were used as negative and positive control, respectively.

Among the 181 isolates studied, 82 (45.3%) were identified as *S. aureus* and 99 (54.7%) were classified as CNS: 27 (14.9%) *Staphylococcus chromogenes*, 26 (14.4%) *Staphylococcus epidermidis*, 17 (9.4%) *Staphylococcus saprophyticus*, 6 (3.3%) *Staphylococcus warneri*, 6 (3.3%) *Staphylococcus simulans*, 6 (3.3%) *Staphylococcus haemolyticus*, 2 (1.1%) *Staphylococcus xylosum*, 4 (2.2%) *Staphylococcus hominis*, and 5 (2.8%) *Staphylococcus hyicus*.

Interpretation of the results according to the breakpoints defined by the CLSI (2015) revealed a rate of 33 (18.2%) of *Staphylococcus* spp. resistant to oxacillin (Table 1). The MIC₅₀ (concentration that inhibits 50% of isolates tested) was 0.25 $\mu\text{g/mL}$ and the MIC₉₀ (concentration that inhibits 90% of isolates tested) was 1.5 $\mu\text{g/mL}$ (Table 2). In contrast, for vancomycin, *S. aureus* exhibited MIC₅₀ and MIC₉₀ values of 0.5 and 1.5 $\mu\text{g/mL}$, respectively. In CNS, the MIC₅₀ and MIC₉₀ values were 1.0 and 1.5 $\mu\text{g/mL}$, respectively. All *Staphylococcus* spp. analyzed were susceptible to vancomycin.

The *mecA* gene was detected in 8 (4.4%) CNS isolates; 2 did not exhibit phenotypic resistance (Table 3). All isolates carrying the *mecA* gene belonged to the species *S. epidermidis* and originated from Paraná, Santa Catarina, or Minas Gerais. The *mecA* gene was not detected in the remaining isolates, although 27 (14.9%) strains were resistant by the phenotypic method. Thus, the presence of the *mecA* homolog *mecC* was investigated. A PCR product with a size similar to that expected for this gene was detected in 12 of these 27 isolates. To confirm the presence of *mecC*, the 12 isolates were sent for sequencing. Sequence analysis showed that the products were not *mecC* but a gene with 99% identity to *mecA*, which was considered an ancestor of the gene (GenBank accession number: AB547234.1). Five (62.5%) of the 8 isolates analyzed were classified as SCC*mec* type I. The other 3 (37.5%) isolates were classified as type IV.

Regarding β -lactamase production, 174 (96.13%) of the 181 isolates tested were positive. Among the negative isolates, only 2 (2.5%) were *S. aureus*, whereas the other 5 (4.85%) were identified as CNS. Hyperproduction of β -lactamase was evaluated in isolates that were resistant to oxacillin by the phenotypic method but that tested negative for detection of the *mecA* and *mecC*

Table 1. Phenotypic resistance (number of isolates) to oxacillin and vancomycin, vancomycin heteroresistance, and hyperproduction of β -lactamase in *Staphylococcus* spp. isolated from bovine subclinical mastitis

Species	Resistance to oxacillin	Resistance to vancomycin	Vancomycin heteroresistance	β -lactamase hyperproduction
<i>S. aureus</i>	1	0	1	2
<i>S. chromogenes</i>	5	0	0	4
<i>S. epidermidis</i>	9	0	5	3
<i>S. haemolyticus</i>	1	0	4	1
<i>S. saprophyticus</i>	10	0	2	9
<i>S. simulans</i>	3	0	0	3
<i>S. xylosus</i>	2	0	0	2
<i>S. warneri</i>	2	0	1	2
Total	33	0	13	26

genes. β -Lactamase hyperproduction was detected in 26 (96.3%) of the 27 isolates tested. The species with the largest number of hyperproducers was *S. saprophyticus*, with 9 (33.3%) isolates (Table 1).

Thirteen (7.18%) of the 181 isolates grew on BHI agar plates containing 4 or 6 $\mu\text{g/mL}$ vancomycin. Seven isolates that grew at the lower concentration also exhibited growth when the concentration was increased to 6 $\mu\text{g/mL}$ (Table 1). The following species exhibited heteroresistance: *S. epidermidis* ($n = 5$), *S. haemolyticus* ($n = 4$), *S. saprophyticus* ($n = 2$), *S. warneri* ($n = 1$), and *S. aureus* ($n = 1$).

Thirty-three (18.2%) isolates were resistant to oxacillin by the phenotypic method, but only 8 (4.4%) of these isolates carried the *mecA* gene. The remaining isolates may have developed resistance through other mechanisms, such as hyperproduction of β -lactamase (Geha et al., 1994). This result demonstrates the need to use phenotypic and genotypic methods to detect resistance. Investigation of SCCmec types in the *mecA*-positive isolates revealed the presence of cassette type I in 5 isolates, whereas 3 isolates carried type IV. Both cassette types (I and IV) carry the *mecA* gene as the single resistance determinant, conferring resistance only to this class of antimicrobials (Boyle-Vavra and Daum, 2007).

Staphylococcal cassette chromosome *mec* typing is an important tool for the understanding of the epidemiology and clonal relationship of methicillin-resistant

Staphylococcus aureus (MRSA), which can be classified into hospital-associated (HA)-MRSA, community-acquired (CA)-MRSA, and livestock-associated (LA)-MRSA. Substantial differences exist between these types in terms of the distribution of toxins, risk factors, and antimicrobial susceptibility (Vandenesch et al., 2003). The SCCmec types IV, V, and VII are frequently associated with CA-MRSA, which carry a smaller cassette that confers resistance only to β -lactam antimicrobials. In contrast, HA-MRSA frequently harbor type I, II, or III and are usually resistant to multiple drugs (Vandenesch et al., 2003), and LA-MRSA frequently harbor SCCmec type XI and are found in a variety of *Staphylococcus* spp. isolated from different animal species (Stefani et al., 2012).

The presence of the *mecA* gene in 2 isolates that were susceptible to oxacillin by the phenotypic method, both carrying SCCmec type IV, suggests the lack of phenotypic expression of oxacillin resistance or the presence of heterogeneous resistance. The same was reported in a study conducted in China (Pu et al., 2014) in which 49 isolates carried *mecA* and 37 were susceptible to oxacillin by phenotypic methods. Taken together, these results suggest the need for genotypic methods to accurately identify oxacillin resistance.

A total of 33 (18.2%) *Staphylococcus* spp. were resistant to oxacillin. Variation in the susceptibility profile of mastitis isolates has been observed between different geographic regions and even between herds of the same

Table 2. Interpretative breakpoints of MIC of staphylococci strains isolated from bovine subclinical mastitis

Species	Oxacillin ¹			Vancomycin ²		
	MIC ₅₀ ($\mu\text{g/mL}$)	MIC ₉₀ ($\mu\text{g/mL}$)	Resistant (no.)	MIC ₅₀ ($\mu\text{g/mL}$)	MIC ₉₀ ($\mu\text{g/mL}$)	Resistant (no.)
<i>Staphylococcus aureus</i>	0.094	0.25	1	0.50	1.0	0
CNS	0.25	1.5	32	1.0	1.5	0

¹Oxacillin breakpoints: *S. aureus*: susceptibility ≤ 2 $\mu\text{g/mL}$; resistance ≥ 4 $\mu\text{g/mL}$; CNS: susceptibility ≤ 0.25 $\mu\text{g/mL}$; resistance ≥ 0.5 $\mu\text{g/mL}$ (CLSI, 2015).

²Vancomycin breakpoints: *Staphylococcus* spp.: susceptibility < 4 $\mu\text{g/mL}$; resistance ≥ 4 $\mu\text{g/mL}$ (CLSI, 2015).

Table 3. Comparison of the presence of the *mecA* gene by PCR with the phenotypic method

PCR	Phenotypic method: Oxacillin (1 µg/mL)			
	Susceptible		Resistant	
	No.	%	No.	%
<i>mecA</i> +	2	1.1	6	3.3
<i>mecA</i> –	146	80.6	27	14.9
Total	148	81.7	33	18.2

region (Owens et al., 1988). The present study instead showed that, despite the widespread use of antimicrobial agents for mastitis treatment, most isolates were susceptible to the antimicrobials tested. Additionally, the MIC of oxacillin and vancomycin were lower for *S. aureus* than for CNS.

Regarding the screening method for detecting vancomycin-heteroresistant *Staphylococcus* spp., our results showed that 7.1% of the isolates were positive in medium containing 4 or 6 µg/mL of the antibiotic. Of these positive isolates, 92.3% belonged to the group of CNS. Hiramatsu (2001) suggested that, if growth is observed on BHI agar containing 4 µg/mL vancomycin, the strain can already be considered heteroresistant to vancomycin. According to Natoli et al. (2009), the heteroresistance phenotype of these isolates may be associated with treatment failure or may be a precursor of glycopeptide resistance. A study carried out in Turkey analyzed 100 strains of staphylococci from cases of bovine mastitis; 10 strains were found to have reduced susceptibility to vancomycin with a MIC of 4 to 8 µg/mL. A PCR was performed for the detection of the *vanA* gene and the results showed that the strains did not carry this gene. Because the *vanA* gene was not present, the mechanism for heteroresistant strains could be explained by changes in the cell wall biosynthesis of the strains (Pehlivanoglu and Yardimci, 2012). The clinical significance of heteroresistance is not fully understood, and a limiting factor in our understanding is the small number of studies involving bovine mastitis isolates.

Regarding vancomycin heteroresistance, the association of these results with those of phenotypic resistance to oxacillin and presence of the *mecA* gene showed that only isolates carrying SCC_{mec} type I exhibited heteroresistance. Although the mechanism of vancomycin resistance has not been studied in these heteroresistant subpopulations, with the confirmation of the presence or absence of the *van* genes, this resistance is probably due to thickening of the bacterial cell wall, preventing entry of the antibiotic into the cell (Billot-Klein et al., 1996).

In the present study, 174 (96.13%) of the isolates produced β-lactamase. In addition, 26 (96.3%) of the 27 oxacillin-resistant isolates that did not carry the *mecA* or *mecC* gene were confirmed to be hyperproducers of β-lactamase. These findings indicate that the presence of these β-lactamase producers may favor the survival of other antibiotic-susceptible microorganisms in an infectious process (Tavares, 2001).

The present results demonstrate the need to evaluate the microbiological and molecular aspects of bovine mastitis isolates because the presence of the *mecA* gene in *S. epidermidis* confirms that cow milk can harbor resistant strains, even though these strains may have come from humans.

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