Antimicrobial resistance and detection of \textit{mecA} and \textit{blaZ} genes in coagulase-negative \textit{Staphylococcus} isolated from bovine mastitis

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ABSTRACT.- Soares L.C., Pereira I.A., Pribul B.R., Oliva M.S., Coelho S.M.O. & Souza M.M.S. 2012. Antimicrobial resistance and detection of \textit{mec} and \textit{blaZ} genes in coagulase-negative \textit{Staphylococcus} isolated from bovine mastitis. \textit{Pesquisa Veterinária Brasileira} 32(8):692-696. Departamento de Microbiologia Veterinária, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, BR 465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: miliane@ufrrj.br

The present study evaluated the pheno- and genotypical antimicrobial resistance profile of coagulase-negative \textit{Staphylococcus} (CNS) species isolated from dairy cows milk, specially concerning to oxacillin. Of 100 CNS isolates, the \textit{S. xylosus} was the prevalent species, followed by \textit{S. cohnii}, \textit{S. hominis}, \textit{S. capitis} and \textit{S. haemolyticus}. Only 6% were phenotypically susceptible to the antimicrobial agents tested in disk diffusion assay. Penicillin and ampicillin resistance rates were significantly higher than others antimicrobials. Four isolates were positive to \textit{mecA} gene (4%), all represented by the \textit{S. xylosus} species. The \textit{blaZ} gene was detected in 16% of the isolates (16/100). It was noticed that all \textit{mecA} + were also positive to this gene and the presence of both genes was correlated to phenotypic beta-lactamic resistance. We conclude that CNS species from bovine milk presented significantly distinct antimicrobial resistance profiles, evaluated by phenotypic and genotypic tests, which has implications for treatment and management decisions.

INDEX TERMS: Mastitis, coagulase negative staphylococci, antimicrobial resistance, gene \textit{mecA}, gene \textit{blaZ}, cattle.

RESUMO.- [Resistência antimicrobiana e detecção dos genes \textit{mec} e \textit{blaZ} em \textit{Staphylococcus} coagulase-negativos isolados de mastite bovina.] O presente estudo avaliou o perfil fenogenotípico de resistência aos antimitóricos em espécies de \textit{Staphylococcus} coagulase-negativo (ECN) isoladas do leite de vacas com mastite, em especial considerando a oxacilina. Dos 100 isolados de ECN, \textit{S. xylosus} foi a espécie predominante, seguida por \textit{S. cohnii}, \textit{S. hominis}, \textit{S. capitis} and \textit{S. haemolyticus}. Apenas 6% dos isolados foram fenotipicamente suscetíveis aos agentes antimitóricos testados no ensaio de difusão em disco. O percentual de resistência à penicilina e ampicilina foi significativamente maior que aos outros antimitóricos. Quatro isolados foram positivos para o gene \textit{mecA} (4%), sendo todos representados pela espécie \textit{Sxylosus}. O gene \textit{blaZ} foi detectado em 16% dos isolados (16/100), sendo todos \textit{mecA} positivos e a presença de ambos os genes foi correlacionada com a resistência fenotípica aos beta-lactâmicos. Foi possível concluir que as espécies de ECN provenientes de leite bovino apresentaram distintos perfis de resistência antimitóbiana, avaliados por testes fenotípicos e genotípicos, podendo dificultar a adoção de medidas de tratamento e manejo dos animais.


INTRODUCTION

Bovine mastitis is a multifactorial disease that results in reduced milk production, changes in milk composition and...
milk discard. It imposes serious economic losses for the farmers and the dairy industry (Ribeiro et al. 2003, Pitskala et al. 2004). Mastitis can be moderate or severe, and can be caused by many different bacterial species, mainly by Staphylococcus and Streptococcus strains (Pyorala, 2002). Nowadays coagulate-negative staphylococci (CNS) are of great interest in veterinary medicine because they are currently considered emerging pathogens of bovine mastitis. Although CNS are not as pathogenic as the other principal mastitis pathogens and infection mostly remains subclinical, they can cause persistent infections, which result in increased milk somatic cell count (SCC) and decreased milk quality (Pyorala 2002). Prevalent CNS species vary according to the geographical region under scrutiny (Huxley et al. 2002).

Antibiotic resistance is the most puzzling question of public health in the earlier decade of this 21st century. Mastitis is the single most common reason for the use of antimicrobials in dairy cattle husbandry. Use of antimicrobials is required for clinical mastitis, persistent infections and in heifers before calving (Taponen et al. 2006). Therefore, antimicrobial resistance of mastitis pathogens has received much interest over the past few years. Carriage of antimicrobial resistance genes by CNS species in cattle may also be relevant because it potentially poses a human health hazard. It can happens both through lateral transfer of resistance genes between staphylococcal species and through direct transmission of resistant pathogens (Walther & Perreten 2007).

Among the antimicrobial agents approved for use in bovine mastitis, β-lactams, such as penicillins and cephalosporins, play a key role. Resistance to β-lactams in staphylococci is mediated by either β-lactamases codified by blaZ gene or mecA-encoded alternative penicillin binding protein, PBP2a, which shows a reduced binding to β-lactam antibiotics currently available for mastitis therapy. According to recommendations of the CLSI, oxacillin-resistant Staphylococcus isolates shall be reported as resistant to other β-lactam antibiotics (Aarestrup et al. 2006). Humans and dairy cattle may share CNS strains, implying that bovine staphylococcal multidrug resistant might be a zoonotic pathogen. It is difficult to demonstrate the direction of interspecies transmission, but it has been suggested that CNS is more likely to spread from humans to dairy cattle than vice versa (Thorberg et al. 2006).

This study was conducted to determine phenotypic antimicrobial resistance profiles of CNS isolated from bovine milk to antibiotics of clinical relevance in dairy practice with emphasis on oxacillin in order to contribute to the knowledge of circulating CNS.

MATERIALS AND METHODS

Sampling and Bacterial isolation and phenotypic identification

Twenty five dairy cattle farms located in six different towns comprising an important milk production region of the State of Rio de Janeiro, Brazil, were selected due to its medium size, mechanical milking system and veterinary management. A total of 450 animals were evaluated by California Mastitis Test and 228 cows were positive for subclinical mastitis. Individual mammary quarter milk samples were aseptically collected into sterile vials immediately before milking, after discarding the first three milking streams. The milk samples were transported to laboratory and procedures of isolation and identification were performed following Koneman et al. (2008).

The isolates were initially identified by the Gram staining, catalase test and susceptibility to 0.04 U bacitracin (CECON, São Paulo, Brazil) to characterize the genus Staphylococcus (Ban-nerman 2003). Twelve tests were used as following: coagulase, nitrate reduction, urease production and acid production from D-trehalose, sucrose, xilose, α-lactose, D-mannitol, fructose, maltose and D-mannose. CNS isolates from our collection that were previously characterized were used as controls in assays.

Phenotypic antimicrobial tests

The inoculum was obtained from overnight broth cultures and adjusted to achieve approximately 5×10⁵ CFU/ml considering a turbidity equivalent to a 0.5 McFarland standard (CLSI, 2010). Disk diffusion test was employed to determine the susceptibility of penicillin (10UI), ampicillin (10μg), oxacillin (1μg), ampicillin-sulbactam (10/10μg), cefalotin (30μg), vancomycin (30μg), gentamicin (10μg), tetracillin (30μg), enrofloxacin (10μg), and trimethoprim-sulfamethoxazole (1.25/23.75μg) (SENSIFAR-CEFAR®) agents. Strains resistant to above two antimicrobial classes were considered multiresistant. Multiresistant Staphylococcus aureus ATCC43300 was used as positive control (CLSI, 2008). Controle do teste de antibiograma. Que ATCC foram utilizadas?

Oxacillin susceptibility tests comprised disk diffusion with oxacillin (SENSIFAR-CEFAR® - 1mg) and cefoxitin discs (SENSIFAR-CEFAR®, 30mg). Also Minimal Inhibitory Concentrations (MICs) of oxacillin were determined by agar and broth dilution methods where oxacillin concentrations ranging from 0.25 to 256μg/mL were added to Mueller-Hinton (Difco, Detroit, MI, USA) supplemented with 2% of NaCl and MICs were recorded after 24h of incubation at 35°C . Importante informar em que temperatura o teste foi realizado. The MIC used to predict mecA presence was 8 μg/ml (Kohner et al, 1999). Resistance to oxacillin was also evaluated by agar screening using Mueller-Hinton agar supplemented with 4% of NaCl and 6μg/mL of oxacillin. The plates were examined carefully for evidence for small colonies (<1 colony) indicating oxacillin resistance. All the results were interpreted according to CLSI (2010) recommendations.

For beta-lactamase production it was used a nitrocefin disk test (Oxoid®) according to the manufacturer’s instructions.

PCR amplification

DNA was extracted by the lisostaphin method as previously described (Coelho et al. 2009). Polymerase chain reaction for detection of genes mecA (513 bp) and blaZ (639 bp) were carried out using the following primers: 5’ AAA ATC GAT GGT AAA GGT TGG C 3’ AGT TCT GCA GTA CGG GAT TTG C and 5’ TAC AAC TGT AAT ATC GGA GGG 3’ CAT TAC ACT CTT GCG GGT TTC, respectively. Amplification cycles for mecA were done according to Coelho et al. (2009) considering 40 cycles of 94°C 30s, 55°C 30s, 72°C 1 min with a final extension of 72°C 5 min. The blaZ amplification was performed according Rosato et al (2003) considering a initial denaturation of 94°C 5min followed by 35 cycles of 94°C 30s, 55°C 30s, 72°C 30s. with a final extension of 72°C 5 min. Staphylococcus aureus ATCC43300 mecA + and blaZ + was used as strain control (CLSI, 2008).

The amplicons were evaluated by agarose gel electrophoresis followed by staining in etidium bromide (0.5μg/mL), visualized on UV transilluminator and documented by the program.
antibiotic susceptibility test

Results of the in vitro disk diffusion assays of CNS susceptibility are summarized in Table 1. A highest resistance was observed against penicillin, ampicillin and tetracycline. Associations of ampicillin-sulbactam and trimethoprim-sulfamethoxazole were the most effective antibiotics. It was also detected multiresistant isolates considering resistance above two antimicrobial classes (Table 2). Nine different resistance patterns were identified among the 100 strains. The most frequently observed pattern of multiple resistance was the penicillin-ampicillin-oxacillin-tetracyclin combination, found in 23 (23%) strains.

Oxacillin susceptibility phenotypic testing

All 100 CNS was analysed for oxacillin susceptibility by disc diffusion, broth and agar microdilution, agar screening and cefoxitin disc test following CLSI standards (2010). These susceptibility tests revealed 15 distinct profiles shown in Table 3. Broth microdilution yielded the highest level of resistant strains (40%).

mec and bla genes detection

The mecA gene was detected in 13.8% (4/29) of oxacillin resistant CNS, represented strictly by Staphylococcus xylosus. The blaZ gene was detected in 55% (16/29) of the CNS isolates, all of them presented resistance in nitrocefin test.

<table>
<thead>
<tr>
<th>Profile (number of resistant isolates)</th>
<th>Tests*</th>
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<tbody>
<tr>
<td>1 (40)</td>
<td>MC</td>
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<tr>
<td>2 (11)</td>
<td>-</td>
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<tr>
<td>3 (9)</td>
<td>DM,AS</td>
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<tr>
<td>4 (8)</td>
<td>DD,AS,MP,MC</td>
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<tr>
<td>5 (7)</td>
<td>DM,MC</td>
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<tr>
<td>6 (6)</td>
<td>DD,DM,AS,MP,MC</td>
</tr>
<tr>
<td>7 (5)</td>
<td>DD,AS,MC</td>
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<tr>
<td>8 (4)</td>
<td>DD,DM,MP,MC</td>
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<tr>
<td>9 (2)</td>
<td>DM,MP,MC</td>
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<td>10 (2)</td>
<td>DD,DM,AS,MC</td>
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<tr>
<td>11 (2)</td>
<td>DD</td>
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<tr>
<td>12 (1)</td>
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<td>13 (1)</td>
<td>DM</td>
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<td>14 (1)</td>
<td>DD,MC</td>
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<tr>
<td>15 (1)</td>
<td>DD,DM,MC</td>
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*DD = disk diffusion, MD = modified disk diffusion, AS = agar screen, MP = agar microdilution, MC = broth microdilution.

**RESULTS**

Identification of coagulase-negative Staphylococcus spp.

A total of 100 CNS isolates was obtained from the milk samples evaluated, included 70% of S.xylosus, 10% S. cohnii, 8% S. hominis, 7% S. capitis and 5% Staphylococcus haemolyticus.

**Antibiotic susceptibility test**

Antimicrobial resistance profiles differed significantly between CNS species from milk of dairy cattle (Sampimon et al. 2009). In the present work, the highest resistance rate was observed against penicillin, ampicillin and tetracyclin. Penicillin and ampicillin resistance in Staphylococcus spp. is a worldwide phenomenon, with increasing prevalence, despite the selective pressure enforced by the improper use of beta-lactams in mastitis treatment (Bonna et al. 2007). The emergence of high levels of penicillin resistance followed by the development and spread of strains resistant to the semisynthetic penicillins (methicillin, oxacillin, and nafcillin), macrolides, tetracyclines, and aminoglycosides has made the therapy of staphylococcal disease a global challenge. The tetracycline resistance detected can be attributed to the semisynthetic penicillins (methicillin, oxacillin, and nafcillin), macrolides, tetracyclines, and aminoglycosides has made the therapy of staphylococcal disease a global challenge.
distributed to the large use of this antibiotic in mastitis treatment and in the water of the herd as a prophylactic measure aimed at reducing infections (Booth & McDonald 1992). It was detected that the association of an antibiotic and a beta-lactamase inhibitor, ampicillin + sulbactam and a nucleic acid synthesis inhibitor, trimethoprim + sulfamethoxazole were the most effective antibiotics. In human clinical cases, the use of beta-lactamase inhibitor is an alternative in case of beta-lactam resistant bacteria (Gentilini et al. 2002). In animal production system it does not worthy because of its high cost. Otherwise, the efficiency of a beta-lactamase inhibitor associated with the detection of blaZ gene in 55% of oxacillin-resistant strains and positive results obtained from nitrocefin test gives us a clue that beta-lactamases must be greatly implicated in the detected resistance. The widespread use of antibiotics has been responsible for the emergence of multidrug resistant bacteria. Antibiotic use in subtherapeutic levels as growth promoters is still common in Brazilian animal production including dairy farms. In the present study, it was observed multiresistance from two to four tested antimicrobial classes. In their report, Machado et al. (2008) detected that all CNS isolates from bovine mastitis showed resistance to two or more antimicrobial agents.

During recent years, methicillin resistance in animals has gained particular attention from public health authorities. It is known that the use of antimicrobials for mastitis treatment can promote emergence or survival of MRSA (Staphylococcus aureus methicillin resistant) and others methicillin resistant staphylococci in dairy cattle (Moon et al. 2007, Garcia-Alvarez et al. 2009). It has also been hypothesized that MRCCS (mecillin resistant coagulase negative Staphylococcus) of agricultural animals may serve as important reservoirs for the transfer of antimicrobial resistance genes. Nevertheless, little information is available on methicillin-resistant CNS (MRCCS) from dairy cattle (Febler et al. 2010).

As methicillin resistance and multidrug resistance in bovine CNS have been described before (Sawant et al. 2009, Fessler et al. 2010) further evaluations of oxacillin resistance were performed by different methods. Since oxacillin maintains its activity during storage better than methicillin, laboratory diagnosis of methicillin resistance is based on the testing of oxacillin. Accurate detection of oxacillin/methicillin resistance can be difficult due to the possible coexistence of two subpopulations (susceptible and resistant) within a culture termed heteroresistance. It is a problem for clinical laboratory personnel because cells expressing resistance may grow slowly than the susceptible population. This heterogeneity can lead to failure of treatment due to false appearance of susceptibility. In this study, the highest resistance rate from oxacillin susceptibility assays was detected in the broth microdilution. Despite its sensitivity, it is a very laborious and subjective assay, so veterinary CLSI (2008) recommends agar screen and disk diffusion as standard tests for oxacillin evaluation in animal samples.

Although a low level of mecA gene was detected, methicillin resistance can be attributed to other mechanisms like inactivation of oxacillin by increased production of beta-lactamase, codified by blaZ gene. In the present work, a significant blaZ gene prevalence was observed in oxacillin resistant strains. Also all blaZ isolates were mecA+.

CONCLUSIONS

This study showed high incidence of CNS from bovine mastitic milk, specially Staphylococcus xylosus, what is not so commonly reported in literature. Beta-lactam resistance was found to be widely spread among isolates. Fifty one isolates were found to be multiresistant and this finding is rather relevant because of the importance of this antimicrobial class in dairy industry. The susceptibility of oxacillin-resistant strains to beta-lactames inhibitor association pointed to the implication of beta-lactamase production in the detected resistance. BlaZ+ mecA+ strains were resistant to all evaluated beta-lactamics.

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REFERENCES


