Antimicrobial resistance and detection of \(mecA\) and \(blaZ\) genes in coagulase-negative \(Staphylococcus\) isolated from bovine mastitis\(^1\)

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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 \(mec\) and \(blaZ\) genes in coagulase-negative \(Staphylococcus\) isolated from bovine mastitis. 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 \(Staphylococcus\) (CNS) species isolated from dairy cows milk, specifically concerning to oxacillin. Of 100 CNS isolates, the \(S.\) \(xylosus\) was the prevalent species, followed by \(S.\) \(cohnii\), \(S.\) \(hominis\), \(S.\) \(capitis\) and \(S.\) \(haemolyticus\). 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 \(mecA\) gene (4%), all represented by the \(S.\) \(xylosus\) species. The \(blaZ\) gene was detected in 16% of the isolates (16/100). It was noticed that all \(mecA\) + 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 \(mecA\), gene \(blaZ\), cattle.

INTRODUCTION

Bovine mastitis is a multifactorial disease that results in reduced milk production, changes in milk composition and...
Antimicrobial resistance and detection of mec and blaZ genes in coagulate-negative Staphylococcus isolated from bovine mastitis

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 antimicrobial treatment is required for clinical mastitis, persistent infections and in heifers before calving (Tapponen 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-lactams 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 pheno-genotypic 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- neman 2003). Twelve tests were used as following: coagulase, nitrate reduction, urease production and acid production from D-trehalose, sucrose, xiloce, a-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 5x10⁸ 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), tetraciclina (30µg), enrofloxacin (10µg), and trimethoprim-sulfamethoxazol (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. O MIC usado para predizer mecA presença foi 8 µg/ml (Kohn 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 TGG C and 5’ TAC AAC TGT AAT ATG GGA GGG 3’ CAT TAC ACT CTT GGC 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 5min. 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 ethidium bromide (0.5mg/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 tetracyclin. Associations of ampicillin+sublactam 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 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.

DISCUSSION
We detected Staphylococcus xylosus as the predominant specie. Despite variations between herds and countries, others CNS i.e. S. chromogenes, S. simulans and S. epidermidis, in general, appear to be the most frequently isolated CNS from mammary secretion samples worldwide (De Vliegher et al. 2003, Taponen et al. 2006). Identification to species level would be important if it reflects differences in virulence characteristics or epidemiology, e.g. in somatic cell count elevation (Sampimon et al. 2009), persistence (Taponen et al. 2006) or transmissibility, and if it has impact on management and treatment decisions. Correct species identification is important for mastitis control and in epidemiological investigations, as well as in understanding of the significance of infections caused by different CNS species.

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 tetracycin. Penicillin and ampicillin resistance in Staphylococcus spp. is a worldwide phenomeno, 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 at-
tributed 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, trimethoprin+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 MRCCNS (methicillin 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 (MRCCNS) 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 mecaA 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 mecaA+.

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+ mecaA+ strains were resistant to all evaluated beta-lactamics.

Acknowledgements.- This study was funded by CNPq and FAPERJ (E-26/171.360/2005, Proc. E-26/171.366/2006, E-26 /110.910/2008).

REFERENCES


