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Short communication: Antimicrobial resistance and virulence characterization of methicillin-resistant staphylococci isolates from bovine mastitis cases in Portugal

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ABSTRACT

Methicillin-resistant staphylococci (MRS) have already been reported as mastitis agents. Such bacterial species are a public health concern, and the characterization of their antimicrobial resistance and virulence profile is important to better control their dissemination. The present work evaluated the distribution of methicillin-resistance among 204 staphylococci from clinical (n = 50) and subclinical (n = 154) bovine mastitis. The presence of the *mecA* gene was determined by PCR. Phenotypic expression of coagulase, DNase, lipase, gelatinase, hemolytic enzymes, and biofilm production was evaluated. The presence of biofilm-related genes, *ica*A, *ica*D, and *bap*, was also determined. Antimicrobial resistance patterns for aminoglycosides, lincosamides, macrolides, fluoroquinolones, sulphonamides, tetracyclines, and fusidic acid were determined. Nineteen (9.3%) isolates were identified as MRS, and the presence of *mec*A in these isolates was confirmed by PCR. Virulence factors evaluation revealed that gelatinase was the most frequently detected (94.7%), followed by hemolysins (73.7%) and lipase (68.4%); 84.2% of the MRS isolates produced biofilm and *ica*A and *ica*D were detected in almost half of the MRS isolates (52.6%), but all were *bap*-negative. Resistance against other antimicrobial agents ranged from 0 (fusidic acid, ciprofloxacin, norfloxacin, enrofloxacin) to 100% (nalidixic acid). Resistance to nalidixic acid and nalidixic acid-tetracycline were the most common antimicrobial resistance profiles (31.6%). This study confirms that despite the low prevalence of MRS, isolates frequently express other virulence traits, especially biofilm, that may represent a serious challenge to clinicians.

Key words: staphylococci, biofilm, methicillin resistance, bovine mastitis

Short Communication

In dairy farms, mastitis remains an important disease with high economic effect. Although many microorganisms have been implicated with this disease, *Staphylococcus aureus* has been described as a major pathogen responsible for bovine mastitis; whereas *Staphylococcus epidermidis* is being increasingly isolated from clinical and subclinical mastitis (Pyörälä and Taponen, 2009). Since the introduction of methicillin in clinical practice in the 1960s, the increasing resistance of staphylococci to β-lactamic antimicrobial compounds has become a major problem. The first methicillin-resistant *Staphylococcus aureus* (**MRSA**) from animal origin was isolated in 1975 from milk samples obtained from cows with mastitis (Kawano et al., 1996). More recently, MRSA and methicillin-resistant *Staph. epidermidis* are frequently isolated from cattle (Walther and Perreten, 2007). Nevertheless, infection due to these bacterial species is rare, and MRSA reports related with bovine mastitis are scarce (Juhász-Kaszanyitzky et al., 2007).

However, methicillin-resistant staphylococci (**MRS**) may be a serious concern for dairy production, as they can express a wide range of virulence traits including surface proteins, exoenzymes, and extracellular toxins, which may strongly influence infection prognosis (Gordon and Lowy, 2008). Biofilm, a recognized virulence factor in staphylococci, has been proposed as a significant element in the persistence of IMI (Tremblay et al., 2013). The intercellular adhesion (*ica*) locus is responsible for the production of a polysaccharide intercellular adhesin and a capsular polysaccharide, 2 major components of staphylococci biofilms. Among the *ica* genes, *ica*A and *ica*D play a key role in biofilm formation (Arciola et al., 2001). *Staphylococcus aureus* strains harboring the biofilm-associated protein (*bap*) gene are found to be strong biofilm producers (Vancraeynest et al., 2004).

The goal of this study was to characterize the antimicrobial resistance profile and virulence traits' distribution among MRS isolates, from a collection of bovine

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clinical and subclinical mastitis staphylococci isolates (n = 204) gathered from 2004 to 2010 belonging to the Immunology and Microbiology Laboratory of the Faculty of Veterinary Medicine, University of Lisbon. Samples were collected from dairy cows belonging to commercial dairy farms located in the Ribatejo-Oeste area of Portugal. Collection of milk samples of bovine subclinical and clinical mastitis and staphylococci isolation were performed according to the National Mastitis Council protocols (National Mastitis Council, 1987). Milk samples of approximately 5 mL were collected aseptically. A volume of 10 μL was plated on Columbia agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) and isolation was performed when colonies of similar morphology were in a number higher that 500 cfu/mL.

Screening of methicillin resistance was performed by the disc diffusion method using oxacillin discs (1 μg; Oxoid, Basingstoke, United Kingdom), according to Clinical and Laboratory Standards Institute (**CLSI**) guidelines (CLSI, 2008). Results were confirmed by the MIC method using oxacillin (0.25–4 μg/mL, Sigma-Aldrich, St. Louis, MO), as recommended by CLSI guidelines. Test performance was monitored using *Staph. aureus* ATCC 25923 and performed in duplicate.

Confirmation of methicillin resistance was performed by conventional PCR targeting the *mec*A gene, using the protocol described in Pereira et al. (2010). The MRSA control strain was kindly provided by Constança Pomba (Faculdade de Medicina Veterinária da Universidade de Lisboa, Portugal). The DNA template for PCR amplification was obtained from a single colony of an overnight culture (18 h, 37°C), using the guanidine thiocyanate method (Merck, Darmstadt, Germany; Pitcher et al., 1989).

Identification of bacterial species was confirmed by PCR according to Pereira et al. (2010) for *Staph. aureus* and *Staph. epidermidis*. Other staphylococci were confirmed for genus using the protocol described by Martineau et al. (1998). *Staphylococcus epidermidis* ATCC 12228 and *Staph. aureus* CECT 4513 were used as PCR amplification controls.

Antimicrobial coresistance profiles were determined using the disc diffusion method according to CLSI guidelines, for aminoglycosides [gentamicin (10 μg), kanamycin (30 μg), neomycin (10 μg; Oxoid), streptomycin (10 μg; Oxoid), tobramycin (10 μg)], lincosamides [clindamycin (2 μg)], macrolides [erythromycin (15 μg)], fluoroquinolones [ciprofloxacin (5 μg), enrofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg)], sulphonamides [sulphamethoxazole-trimethoprim (25 μg)], tetracyclines [tetracycline (30 μg)], and steroid antibacterials [fusidic acid (10 μg)]. All discs were purchased from Oxoid; *Staph. aureus* ATCC 25923 was used as a control for test performance.

All isolates identified as MRS were characterized for virulence traits by phenotypic methods. This characterization included the determination of coagulase activity (Liofilchem, Roseto degli Abruzzi, Italy; Sperber and Tatini, 1975) and DNase (Liofilchem; Weckman and Catlin, 1957), gelatinase (Liofilchem; Semedo et al., 2003), lipase (Becton Dickinson, Pont de Claix, France), and production of hemolysins (bioMérieux; Pereira et al., 2009). *Staphylococcus aureus* CECT 4513, *Staph. epidermidis* ATCC 12228, and *Staph. aureus* ATCC 25923 were used as controls for good quality standards.

Biofilm-forming ability by MRS isolates was evaluated by both phenotypic and genotypic methods. Phenotypic expression was evaluated using a fluorescence in situ hybridization (**FISH**) protocol (Oliveira et al., 2007). Genes involved in biofilm production, including *ica*A, *ica*D, and *bap*, were identified by conventional PCR (Vasudevan et al., 2003; Vancraeynest et al., 2004). *Staphylococcus epidermidis* ATCC 35984 (RP62A) and *Staph. epidermidis* ATCC 12228 were used as biofilm and nonbiofilm producer control strains, respectively.

Methicillin resistance screening by oxacillin disc diffusion detected 19 (9.3%) MRS, identified as *Staph. epidermidis* (n = 16; 84.2%), *Staphylococcus haemolyticus* (n = 1; 5.3%), *Staphylococcus simulans* (n = 1; 5.3%) and *Staphylococcus chromogenes* (n = 1; 5.3%). These isolates were obtained from cows with bovine clinical (n = 3; 15.8%) and subclinical mastitis (n = 16; 84.2%). Oxacillin MIC results were interpreted according to CLSI guidelines M31-A3 (CLSI, 2008) breakpoints and showed that most isolates (78.9%) presented resistant MIC results ≥4 μg/mL. Only 3 isolates showed different results between disc diffusion and MIC determination. Methicillin resistance was confirmed by *mec*A PCR amplification in all 19 isolates.

Resistance to other antimicrobial agents ranged from 0 (fusidic acid and ciprofloxacin, norfloxacin enrofloxacin) to 100% (nalidixic acid). None of the isolates was susceptible to all antimicrobials tested, whereas the vast majority (16 isolates) showed a multiresistance profile. The most common antimicrobial resistance profiles were resistance to nalidixic acid and nalidixic acid-tetracycline (31.6%; Table 1).

Virulence traits characterization revealed that no isolate was coagulase-positive, 14 isolates produced hemolysins (73.7%), 2 produced DNase (10.5%), 18 were gelatinase-positive (94.7%), and 13 were lipase-positive (68.4%). Biofilm production revealed that 84.2% of the isolates (n = 16) were able to produce biofilm after 24 h of incubation. The PCR amplifications revealed 3 isolates were only positive for *ica*D (15.8%) and 10