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Methicillin-resistant *Staphylococcus aureus* (MRSA) in sheep and goat bulk tank milk from Southern Italy☆

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA), an important widespread cause of severe infection in both humans and animals, is a pathogen of significant public health concern. In this paper the prevalence of MRSA in sheep and goat bulk tank milk from southern Italy was assessed and nasal swabs from people working on the positive farms were collected. MRSA isolates from milk and nasal swabs were characterized by *spa* typing, MLST and SCC*mec* typing and tested for antimicrobial resistance. MRSA was detected in 2 of the 162 (1.23%) bulk tank samples analysed: the first from a sheep farm (Farm 1) and the second from a goat farm (Farm 2). As for farm personnel, no MRSA was detected in the unique nasal swab from Farm 1; while MRSA was detected in all the 3 nasal swabs from Farm 2. The MRSA isolate from Farm 1 was *spa* type t127, ST1, SCC*mec* IVa. The MRSA isolates from bulk tank milk and humans in Farm 2 showed an identical genetic profile: *spa* type t1255, ST398, SCC*mec* V. In Farm 2 all the isolates had the characteristics of general multidrug resistance and the milk and human MRSA had the same antimicrobial resistance pattern. Although the results showed low prevalence of MRSA in sheep and goat milk from southern Italy, the detection of recognized zoonotic genotypes in milk of both farms and the isolation in people working in one of the positive farm of MRSA strains with a genetic profile identical to that of MRSA from milk, emphasize the public health concern and highlight the need for additional surveillance including the detection of MRSA.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of hospital associated infections (HA-MRSA: hospital—acquired MRSA) and during the last decade has emerged as a significant pathogen in the community (CA-MRSA: community—associated MRSA) (Pantosti, 2012; Wendlandt et al., 2013). In recent years other MRSA clones, associated with exposure to livestock (livestock associated—LA-MRSA), have emerged in different countries worldwide (Köck et al., 2013; Wendlandt et al., 2013; Witte et al., 2007). In particular, LA-MRSA genetically classified under Clonal Complex 398 (CC 398) seem to have found a reservoir in animals, primarily pigs, cattle and horses and have been shown to be able to colonize and cause serious infections in humans in close contact with these animals (farmers and their relatives, veterinarians) (De Martino et al., 2010; Lozano et al., 2011a,b; Soavi et al., 2010). It has also been demonstrated that the handling/consumption of food of animal origin contaminated by MRSA could provide a potential vehicle for transmission to humans (EFSA, 2009; Feingold et al., 2012). In fact, MRSA clones were largely isolated from meats (Agersø et al., 2012; de Boer et al., 2009; Kitai et al., 2005; Lim et al., 2010; O'Brien et al., 2012; van Loo et al., 2007b), fish (Atyah et al., 2010; Hammad et al., 2012), milk, dairies and ice cream (Karmal et al., 2013; Normanno et al., 2007) from all the world, and often the isolates were considered potentially harmful for consumers.

S. aureus is the most important agent of mastitis in ruminants worldwide. It is reported that MRSA strains can cause intramammary infection in cows, and goats (Aras et al., 2012; Fessler et al., 2010; Pilla et al., 2012; Vanderhaeghen et al., 2010). When these microorganisms cause subclinical infections, they can be transferred to milk without any alteration of the sensorial characteristics of the product, thus spreading through the dairy food chain. Numerous reports have described the prevalence of MRSA in bovine milk (Benedetti et al., 2010; Fessler et al., 2010; Vanderhaeghen et al., 2010) and the transmission of MRSA between people working on farms and dairy cattle (Antoci et al., 2013; Spohr et al., 2011). The

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overall prevalence of MRSA in milk from both healthy and infected cows has been reported low internationally, ranging from 0.3% in the United Kingdom to 9% in Belgium (Paterson et al., 2012; Vanderhaeghen et al., 2010). The occurrence of MRSA in bovine bulk tank milk was investigated in Southern Italy where a prevalence of 2% was found (Parisi et al., 2014). Few studies have investigated the prevalence of MRSA in small ruminants' milk (Aras et al., 2012; Chu et al., 2012; Cortimiglia et al., 2015; Foti et al., 2012; Virdis et al., 2010), although the consumption of dairy products made from the raw milk of these animal species is very common/widespread in the population, primarily in Mediterranean countries (Greece, Spain, Italy). In Italy many traditional goat or sheep cheeses (Pecorino, Cacioricotta etc) are made exclusively from unpasteurized milk (Albenzio et al., 2001; Spanu et al., 2014; Tofalo et al., 2015) and their consumption is very high, above all in those Italian regions (Southern Italy, Sicily and Sardinia) where sheep and goat farming is diffused.

The aim of this study was to (1) assess the occurrence of MRSA in sheep and goat bulk tank milk from southern Italy; (2) characterize the MRSA isolates collected from bulk tank milk; (3) collect nasal swabs from humans in the farms where MRSA was detected in milk; and (4) compare the MRSA isolates from bulk tank milk with the human isolates.

2. Materials and methods

2.1. Collection of samples

One hundred and sixty two bulk tank milk samples were collected between January and July 2014 from sheep and/or goat farms located in two regions of Southern Italy (Apulia and Basilicata). Samples were distributed as follows: 96 were sheep milk, 49 were goat milk, 17 were mixed sheep and goat milk. The bulk tank milk was stirred before sample collection. One bulk milk sample per farm (50 ml; one replicate per sample) was aseptically collected from the top of the tank and immediately transported, under refrigeration, to the laboratory where it was stored at −80 °C prior to testing. Samples were thawed at room temperature for about one hour before processing. In the farms where MRSA were detected in bulk tank milk, nasal swabs were collected from all consenting humans working with animals. A cotton—tipped swab was collected from both nostrils, kept at 4 °C (not longer than 24 h) in Stuart's medium and transported to the laboratory.

2.2. Isolation of MRSA and confirmation

1 ml of each milk sample or nasal swabs were added to Mueller-Hinton broth (Biolife Italiana, Milano, Italy) supplemented with 6.5% (W/v) NaCl (Sigma–Aldrich, St. Louis MO, USA) and incubated for 24 h at 37 °C. Then, 20 μl of each culture were spread onto a MRSA-SELECT® plate (Bio-Rad, Marnes la Coquette, France) and incubated at 37 °C for 24-48-72 h (Nahimana et al., 2006). Suspected MRSA colonies (pink colonies) detected on MRSA-SELECT® were subcultured on a Columbia Sheep Blood Agar plate (Oxoid, Basingstoke, Hampshire, UK) for purification and confirmed as MRSA using both microbiological and molecular analysis.

2.2.1. Microbiological confirmation

2.2.1.1. Disk diffusion test. Oxacillin and cefoxitin disk diffusion susceptibility tests were performed with 1 μg oxacillin and 30 μg cefoxitin disks (Rosco Diagnostica, Taastrup, Denmark), following the recommendations given by CLSI (Clinical and Laboratory Standards Institute, 2013). Mueller-Hinton agar plates (Biolife) were inoculated with a suspension (equivalent to a 0.5 McFarland standard) of each presumptive MRSA strain. The plates were incubated at 37 °C and zone diameters were read after 18–24 h. Following breakpoints were considered: oxacillin, resistant ≤10 mm, intermediate 11–12 mm, susceptible ≥13 mm; cefoxitin, resistant ≤21 mm, susceptible ≥22 mm (Shariati et al., 2010; Thaker et al., 2013).

2.2.1.2. Agar screening method. The suspension of bacteria (adjusted to match 0.5 McFarland turbidity standard) was inoculated on Oxacillin Salt Screen Agar® (Mueller-Hinton agar containing 4% NaCl and 6 μg oxacillin/ml-Biolife). Plates were incubated at 37 °C for 24 h and any growth on the plate was regarded as resistant to methicillin (Shariati et al., 2010).

2.2.1.3. E-test method. Mueller-Hinton agar plates supplemented with 2% NaCl (Biolife) were inoculated by streaking the standardized inoculums (equivalent to a 0.5 McFarland standard) with sterile swab. Oxacillin *E*-test strips (bioMèrieux, Marcy l'Etoile, France) were placed on the plates, followed by an incubation at 37 °C for 18–24 h. Minimum inhibitory concentration (MIC) for each isolate was read at the intersection point of the zone of growth inhibition with the graduated strip (resistant ≥4 μg/ml; susceptible: ≤2 μg/ml) (Shariati et al., 2010).

2.2.2. Molecular confirmation

Genomic DNA of the presumptive MRSA isolates was extracted using a GenomicPrep® cell and tissue isolation kit (Amersham, Piscataway, NJ, USA) following the manufacturer's instructions. DNA concentration was determined spectrophotometrically and adjusted with distilled water to 10 ng/μl.

2.2.2.1. Real time PCR. Real-time PCR for the detection of the methicillin resistance (*mecA* gene) contained a pair of primers *mec*A147-F and *mec*A147-R previously described (Zhang et al., 2005). Real-time PCR for the detection of *S. aureus*-specific sequence contained a pair of primers *sau1* and *sau2* previously described (Strommenger et al., 2003). Both assays were performed in a 25 μl reaction containing 1 μM of each primer, 200 μM each dATP, dTTP, dGTP, and dCTP (Eppendorf, Hamburg, Germany), 1U of HotMaster Taq DNA polymerase (Eppendorf), 10X HotMaster Taq buffer (Eppendorf), 1X EvaGreen (Biotium, Hayward, CA, USA) and 2 μl template DNA by using an initial step of 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension step at 72 °C for 45 s. It was completed with melting curve program of 50–95 °C with a heating rate of 2.3 °C/min and a continuous fluorescence measurement. In both the microbiological and molecular tests mentioned above, *S. aureus* (ATCC 43300) and *S. aureus* (ATCC 29213) (Biogenetics, St. Cloud, USA) were used as methicillin sensitive and resistant controls, respectively. One isolate per positive sample identified and confirmed as MRSA was subjected to genotypic characterization and antimicrobial susceptibility testing.

2.3. Genotypic characterization of MRSA

2.3.1. MLST analysis

The PCR amplifications of the 7 housekeeping genes were performed using the primers described elsewhere (Enright et al., 2000). DNA sequences were obtained using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and BigDye 3.1 Ready reaction mix (Applied Biosystems) according to the manufacturer's instructions. Sequences were imported and assembled using BioNumerics 7.5 software (Applied Maths, Belgium). Alleles and ST were assigned by submitting the DNA sequences to the *Staphylococcus* MLST database (http://saureus.mlst.net/). Strains were grouped into clonal complexes, defined as groups of profiles differing by no more than one gene from at least one other profile of the group as defined in the MLST database.