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Identification of virulence factors in 16S-23S rRNA intergenic spacer genotyped *Staphylococcus aureus* isolated from water buffaloes and small ruminants

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ABSTRACT

Staphylococcus aureus is an important human and animal pathogen, and is regarded as an important cause of intramammary infection (IMI) in ruminants. *Staphylococcus aureus* genetic variability and virulence factors have been well studied in veterinary medicine, especially in cows as support for control and management of IMI. The aim of the present study was to genotype 71 *Staph. aureus* isolates from the bulk tank and foremilk of water buffaloes (n = 40) and from udder tissue (n = 7) and foremilk (n = 24) from small ruminants. The method used was previously applied to bovine *Staph. aureus* and is based on the amplification of the 16S-23S rRNA intergenic spacer region. The technique applied was able to identify different *Staph. aureus* genotypes isolated from dairy species other than the bovine species, and cluster the genotypes according to species and herds. Virulence gene distribution was consistent with genotype differentiation. The isolates were also characterized through determination of the presence of 19 virulence-associated genes by specific PCR. Enterotoxins A, C, D, G, I, J, and L were associated with *Staph. aureus* isolates from buffaloes, whereas enterotoxins C and L were linked to small ruminants. Genes coding for methicillin resistance, Panton-Valentine leukocidin, exfoliative toxins A and B, and enterotoxins B, E, and H were undetected. These findings indicate that RNA template-specific PCR is a valid technique for typing *Staph. aureus* from buffaloes and small ruminants and is a useful tool for understanding udder infection epidemiology.

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INTRODUCTION

Staphylococcus aureus is a worldwide pathogen causing several severe diseases. In ruminants, it is the most common etiological agent of subclinical and clinical mastitis (Watts, 1988), with relevant losses in the dairy industry, as it reduces milk quality, milk production, and increases the cost of production through increased culling. *Staphylococcus aureus* strains produce several virulence factors such as (1) surface proteins that promote colonization of host tissues, (2) proteins that promote bacterial spread in tissues (e.g., leukocidin, kinases, and hyaluronidase), (3) biochemical properties that enhance their survival in phagocytes (e.g., carotenoids and catalase production), (4) surface factors that inhibit phagocytic engulfment (e.g., capsule, protein A), (5) immunological "target like" (e.g., protein A, coagulase, clumping factor, biofilm production), (6) membrane-damaging toxins that lyse eukaryotic cell membranes (e.g., hemolysins, leukotoxin, and leukocidin), (7) exotoxins that damage host tissues or otherwise provoke symptoms of disease [e.g., staphylococcal enterotoxins SEA to SEQ, toxic shock syndrome toxin-1 (**TSST**-1), and exfoliative toxins (ET)], and (8) mechanisms of resistance to antimicrobial agents.

Some virulence factors are expressed by genes that are located on mobile genetic elements called pathogenicity islands (e.g., TSST and some enterotoxins; Novick, 2003) or lysogenic bacteriophages [e.g., Panton-Valentine leukocidin (**PVL**); Narita et al., 2001] and factors associated with suppressing innate immunity, such as the chemotaxis inhibitory protein and staphylokinase, which are integrated in the bacterial chromosome. All these factors can contribute in differ-

ent ways to *Staph. aureus* pathogenicity and, therefore, influence the management of the disease (Dinges et al., 2000; Peacock et al., 2002). The molecular mechanisms responsible for the disease manifestation are not completely understood but differences in gene content and allelic variations between *Staph. aureus* strains are presumed to influence the pathogenesis of infections in cattle (Peacock et al., 2002). The importance of evaluating the combination of *Staph. aureus* virulence factors has been repeatedly emphasized both in human and veterinary medicine (Jarraud et al., 2002; von Eiff et al., 2004), and knowledge about the genetic variability within different *Staph. aureus* populations would help in the design of efficient therapeutic approaches. Actually, the genotype involved affects the prevalence of *Staph. aureus* and the number of infected quarters within a herd (Fournier et al., 2008). Although staphylococcal virulence factors have been identified in many *Staph. aureus* collections isolated from cases of bovine IMI, it is still unknown which factors are specifically associated with water buffalo and small ruminant IMI. The aim of the present study was to genotype and characterize *Staph. aureus* isolated from the milk of water buffaloes, small ruminants, and from udder tissue of sheep.

MATERIALS AND METHODS

Farm Selection and Isolates Identification

In the context of a pilot project focused on the improvement of welfare and control of SCC in water buffaloes and small ruminants in different areas of Latina County (Italy), 212 herds were sampled with the following distribution: 162 water buffaloes, 37 sheep, and 12 goats. Thirty-eight (18%) of these were positive for *Staph. aureus* in bulk tanks and 18 farms decided to collaborate for further analyses. From those farms [5 sheep (A to E), 2 goats (F to G), and 11 buffaloes (H to T)], a total of 71 *Staph. aureus* isolates, originating from water buffaloes (n = 40), sheep (n = 22), and goats (n = 9), were collected. All isolates were selected and stored at Istituto Zooprofilattico Sperimentale Lazio Toscana (IZSLT, Rome, Italy) Latina section, which is the main reference laboratory for the bacterial analyses on those species in the Lazio Region. All milk samples were collected and kept at 4°C until bacteriological analyses were performed according to the National Mastitis Council (NMC, 1999). A total of 7 sheep udder tissues from herd A were also examined. Parenchymal and cisternal tissues were collected from udder for bacteriological investigations and sections were sampled with a sterile swab that was rotated inside a 2-cm-long cut over a surface of 1 cm^2 and then streaked on a sheep blood agar plate and Baird-Parker agar plates. After incubation of 24 to 48 h at 37°C, suspect colonies were picked up from culture plates according to their cultural features (colony appearance and hemolysis) and subcultured on sheep blood agar. Then, their presumptive identification as *Staph. aureus* was confirmed by a tube coagulase test.

DNA Extraction

The coagulase-positive isolates were inoculated in brain-heart infusion broth (Oxoid Ltd., Basingstoke, UK) and incubated aerobically overnight at 37°C. The DNA was extracted from 1 mL of culture broth (about 10^8–10^9 cfu/mL), following the instructions described in Cremonesi et al. (2006), starting from step 2. The quantity and quality of DNA samples were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The DNA samples were then stored at −20°C until use.

16S-23S rRNA Intergenic Spacer Genotyping

The method of Jensen et al. (1993), rearranged by Fournier and coworkers (2008), was used for RNA template-specific PCR (**RS-PCR**) genotyping. This method is based on the amplification of the 16S-23S rRNA intergenic spacer region. In a total volume of 25 μL, each reaction contained 1× HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany), an 800 n*M* concentration of each primer (G1:GAAGTCGTAACAAGG and L1:CAAGGCATCCACCGT), and 30 ng of DNA. The amplification protocol was 95°C for 15 min, which was followed by 27 cycles at 94°C for 1 min, a 2-min ramp to reach the final temperature, and annealing at 55°C for 7 min. After a further 2-min ramp to reach the final temperature, extension was done at 72°C for 2 min. The PCR products were then analyzed using an Agilent 2100 Bioanalyzer with a DNA 7500 LabChip kit (Agilent Technologies Inc., Palo Alto, CA). For interpretation of the results, 2 patterns were considered different if 2 or more peaks of the electropherogram differed in size. Groupings of the RS-PCR profiles were obtained with the BioNumerics 5.0 software package (Applied Maths NV, Sint-Martens-Latem, Belgium) using the unweighted pair group method cluster analysis.

Molecular Strain Characterization

All DNA samples were analyzed by PCR to determine the presence of genes encoding enterotoxins (from *sea* to *sel*), leukotoxins (*luk*E, *luk*S/F-PV, *luk*E, *luk*D, and *luk*M), acquisition of methicillin resistance (*mec*A) and other virulence genes using primers and protocols described in the literature and listed in Table 1. The