

Bovine *Staphylococcus aureus*: Association of virulence genes, genotypes and clinical outcome

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Abstract

Based on our clinical experience on bovine mastitis, we hypothesized that subtypes of *Staphylococcus aureus* (*S. aureus*) exist which differ in their contagious and pathogenic properties. In order to investigate this hypothesis, we analyzed strains of *S. aureus* isolated from spontaneous intramammary infection (IMI) with their virulence gene patterns and genotypes obtained by PCR amplification of the 16S–23S rRNA intergenic spacer (RS-PCR). The genotypes were then associated with epidemiological and clinical data including 26 herds. The results demonstrated a high association between genotypes and virulence gene patterns as well as between epidemiological and pathogenic properties of *S. aureus*. In particular, genotype B was related to high contagiousity and increased pathogenicity whereas the other types (C, OG) were found with infection of single cows. Because of the high clinical relevance, our results indicate the need to subtype the IMI-associated strains of *S. aureus* in the future.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is the most important etiologic agent of contagious bovine mastitis and is very common in Swiss dairy herds (Schällibaum, 1999). It is able to produce a number of virulence factors such as protein A or leukocidins (Kerro Dego et al., 2002). Protein A is located in the cell wall and captures antibodies (Abs) (Foster and McDevitt, 1994). Opsonizing Abs of the IgG2 class, together with neutrophils, play a major role in the defense against invading pathogens (Burton and Erskine 2003). Leukocidins are staphylococcal bicomponent exotoxins of the family of pore forming toxins (Miles et al., 2001). One of the several components is leukotoxin E. Leukoci-

dins were shown to be cytotoxic for erythrocytes and leukocytes including bovine neutrophils and macrophages (Loeffler et al., 1986). These toxins are considered as virulence factors in bovine mastitis (Younis et al., 2005) and may play an important role in the development of this disease (Loeffler et al., 1988). Another virulence factor is coagulase (Sutra and Poutrel, 1994) which is an exoenzyme and clots plasma by conformational activation of prothrombin (Panizzi et al., 2004).

Recent studies suggest that staphylococcal enterotoxins (SETs) act as virulence factors in cattle (Chang et al., 2005). Among other effects, these superantigens induce in this species the production of interleukin 4 and 10 which activate T_H2 cells leading to reduced clearance of microbial pathogens (Burton and Erskine 2003). The five staphylococcal enterotoxins SEA to SEE as well as the toxic shock syndrome toxin-1 (TSST-1) are well known for several years,

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whereas the toxins SEG, SEH, SEI and SER and the staphylococcal enterotoxin-like superantigens SEIJ to SEIU and SEIU have been reported only recently (Omoe et al., 2005).

Various methods exist to subtype *S. aureus*. Phenotyping such as antibiotic susceptibility testing, or checking for production of SETs (Cenci-Goga et al., 2003) become more and more replaced by DNA based methods. They include pulsed-field gel electrophoresis (PFGE) (Anderson et al., 2006; Haveri et al., 2007), binary typing (Zadoks et al., 2000), multilocus sequence typing (MLST) (Enright et al., 2000) or DNA arrays (Monecke and Ehrlich, 2005). In addition, PCR based methods (PCR: polymerase chain reaction) have been used. The latter include random or specific amplification of polymorphic DNA (Stepan et al., 2004). One method repeatedly used to genotype *S. aureus* is PCR amplification of the 16S–23S rRNA intergenic spacer (RS-PCR) as originally described by (Jensen et al., 1993). Its discriminatory power is very similar to the one of PFGE (Kumari et al., 1997), a proposed reference method in genotyping *S. aureus* (Weller 2000). In contrast to PFGE, the RS-PCR allows a high sample throughput.

Of particular clinical importance is the question concerning the existence of subtypes of *S. aureus* responsible for IMI, which differ with respect to their pathogenic and contagious properties, requiring different strategies towards prevention and treatment of the intramammary infection. In order to investigate this hypothesis, we first started a methodological study to obtain an overview of the different subtypes of *S. aureus* which can be isolated from milk samples of cows with spontaneous IMI. As pathogenicity and contagiousity of a certain strain might depend on its repertoire of virulence factors, we analyzed the isolates for their presence of various virulence genes/polymorphisms by multiple PCRs. Because this type of analysis is laborious and therefore not suitable for field studies requiring the examination of numerous samples, we associated the obtained gene patterns with those of the highly discriminatory and rapid RS-PCR. In a second step, the genotypes were then associated with clinical and epidemiological data from 26 herds to study the contagious and pathogenic properties of the subtypes.

2. Material and methods

2.1. *Staphylococcus aureus* isolates

In total, 291 strains of *S. aureus* were included in this study. All of them were isolated from milk samples of cows with spontaneous *S. aureus* intramammary infection using standard procedures proposed by the National Mastitis Council (NMC, 1999). Identification of *S. aureus* and other mastitis pathogens such as *Staphylococcus* spp. or *Streptococcus* spp. was done according to the guidelines of NMC (1999) which include morphology, biochemical properties, and detection of hemolysis. 81 strains were obtained from individual diagnostic analysis (G strains). Additional 210

strains were isolated from bovine milk samples collected during an epidemiological study on *S. aureus* in cow herds of Switzerland (M strains). In each of the 26 herds investigated, all the lactating cows ($n = 449$) were checked for udder health comprising clinical examination of udder, teats and visual milk inspection. Furthermore, milk of each quarter was checked by the California Mastitis Test (CMT) followed by aseptical sample collection for bacteriological testing and analysis of somatic cell counts (SCC). The samples were transported at 4 °C and analyzed for SCC within 24 h. Samples designed for bacteriology and PCR analysis were stored at –20 °C until further use.

All the 291 isolates were checked by PCR (see below) for the presence of the *nuc* gene which codes for the thermo-nuclease and is known to be specific for *S. aureus* (Brakstad et al., 1992). Isolates lacking this gene were excluded from the present study. All the *nuc*-positive isolates were then subjected to genotype analysis (see below). The 291 isolates further served as a source to create a subgroup of epidemiologically independent strains ($n = 101$). It comprised all the G strains (all the strains were from different herds) and a set of M strains: for each of herd 1 to 59 (see Table 5), 1 representative of the 2 most frequently observed genotypes was included.

2.2. Extraction of nucleic acids

A single colony of *S. aureus* was cultured in 4 ml sterile TS (Trypticase Soy Broth; Becton, Dickinson and Company) at 37 °C overnight. 1 ml of the culture was then centrifuged at $18,000 \times g$ for 10 min. The pellet was resuspended in 950 μ l sterile water and total nucleic acid (NA) containing both DNA and RNA were extracted according to (Chavagnat et al., 2002). The eluate was tested for the quantity and integrity of NA by electrophoresis using a 0.8% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH = 8.3) containing ethidium bromide (0.5 μ g/ml). The gels were visualized and photographed under UV light. The isolated NAs were stored at –20 °C until further use.

2.3. Primers

The PCR primers described by Monday and Bohach (1999) were used to amplify the SET genes *sea* to *sej*. For *tst*, we employed the primers according to Lovseth et al. (2004). The *coa* gene (coagulase) and the α -region of *spa* (protein A) were detected by the primers published by Akineden et al. (2001). Primers for the *nuc* and the *lukE* gene (coding for leukotoxin E) were designed with the OLIGO 6.0 software (National Biosciences Inc.) and are shown in Table 1. Finally, the G1 and L1 primers described by Jensen et al. (1993) were used for genotyping by RS-PCR.

2.4. Analysis of virulence genes and genotyping

The PCR reaction mix (total volume 25 μ l) for the multiplex assays included 1x PCR buffer (Qiagen), 4.0 mM

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