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Mastitis-related subtypes of bovine *Staphylococcus aureus* are characterized by different clinical properties

H. U. Graber,*[1] J. Naskova,* E. Studer,* T. Kaufmann,* M. Kirchhofer,* M. Brechbühl,* W. Schaeren,† A. Steiner,* and C. Fournier*

*Clinic for Ruminants, Department of Clinical Veterinary Medicine, University of Berne, Bremgartenstrasse 109a, PO Box 8466, 3001 Berne, Switzerland

†Agroscope Liebefeld-Posieux, Swiss Federal Research Station for Animal Production and Dairy Products, Schwarzenburgstrasse 161, 3003 Berne, Switzerland

ABSTRACT

Based on a former study from our group, one subtype of *Staphylococcus aureus* was associated with high within-herd prevalence of mastitis, whereas the other subtypes were associated with a low prevalence (sporadic intramammary infection). To confirm this hypothesis, a prospective study was done in 29 Swiss dairy herds. In particular, milk samples were collected from 10 herds with *Staph. aureus* herd problems (cases) and compared with samples from 19 herds with only sporadic cases of with *Staph. aureus* intramammary infection (controls). The isolates were tested for their virulence gene pattern and genotyped by PCR amplification of the 16S–23S rRNA intergenic spacer. The patterns and genotypes were then associated and compared with epidemiological and clinical data. Confirming the hypothesis, one particular subtype (genotype B) was associated with high within-herd and within-cow prevalence of intramammary infection, whereas the other subtypes were associated with low within-herd prevalence and infected single quarters. The gene patterns and genotypes were highly related, demonstrating the genetic diversity of the genotypes. The somatic cell counts were clearly increased in herds with a genotype B problem compared with herds with infections of other genotypes. Based on the different clinical properties and treatment consequences associated with these different genotypes found in Switzerland, we recommend subtyping *Staph. aureus* in other countries to determine if this finding is universally applicable.

Key words: subtyping, mastitic, *Staphylococcus aureus*, diagnostics

INTRODUCTION

Staphylococcus aureus is one of the most important causes of chronic, clinical, or subclinical bovine mastitis worldwide (IDF, 2005) and is associated with great economic losses (Seegers et al., 2003; Kirchhofer et al., 2007) in dairy herds.

In a previous study (Fournier et al., 2008) in Swiss cows, the authors found that *Staph. aureus* isolated from bovine IMI is a genetically heterogeneous group. By PCR amplification of the 16S–23S rRNA intergenic spacer region [ribosomal spacer (**RS**)-PCR], 17 genotypes were detected in 101 epidemiologically independent isolates. Two of the genotypes, genotype B (**GTB**) and genotype C (**GTC**), were common (80.2%), whereas the other 15 genotypes (**GTOG**) occurred rarely, each accounting for 1.0 to 4.0% of all the isolates. The same study further demonstrated that the genotypes were highly associated with their virulence gene pattern (**VGP**). The corresponding patterns were obtained 1) by PCR-testing for the presence of the staphylococcal enterotoxin genes *sea* to *sej* and the *tst* gene (toxic shock syndrome toxin-1, **TSST-1**); 2) by evaluation for polymorphisms of the protein A (*spa*) and the coagulase (*coa*) genes (PCR); and 3) by searching for RFLP of the leukotoxin E gene (*lukE*). In particular, we found that GTB was characterized by the presence of the *sea*, *sed*, and *sej* genes, a long x-region of *spa*, and 3 *lukE* fragments. In contrast, GTC was positive for *sec*, *seg*, and *tst*, a short x-region of *spa*, and 2 *lukE* fragments. The GTOG were heterogeneous in their VGP. The same study (Fournier et al., 2008) further revealed remarkable differences in prevalences of IMI among the 3 genotypes. Considering GTB, up to 64.7% of cows in a herd were infected by strains of this genotype and 49% of the cows had more than 1 quarter infected. In the case of GTC and GTOG, however, IMI was found in 1 to 3 cows of a herd. In all cases, only one quarter within an udder of a cow was infected.

The staphylococcal enterotoxins (**SET**) and TSST-1 comprise a large family of proteins (Omoe et al., 2005) with superantigenic properties (Balaban and Rasooly, 2000). In cattle, they act as virulence factors (Chang et al., 2005) and induce, among other effects, the production of the interleukins 4 and 10, which leads to

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[1]Corresponding author: hans.graber@knp.unibe.ch

reduced clearance of microbial pathogens (Burton and Erskine, 2003). Protein A is associated with the cell wall and captures antibodies inhibiting opsonization of the pathogen repelling the host defense (Foster and McDevitt, 1994). Leukotoxin E is a component of the pore-forming leukocidins, which are cytotoxic for erythrocytes and leukocytes (Miles et al., 2001). Coagulase, an exoprotein, clots plasma fibrinogen by forming a complex with prothrombin (Panizzi et al., 2004).

Based on the study of Fournier et al. (2008), we assumed that the genotypes of *Staph. aureus* are contagious to varying degrees and that GTB accounts for herd problems, whereas GTC and GTOG are associated with sporadic IMI. The confirmation of this hypothesis would be of great clinical relevance to propose type-specific hygiene and treatment strategies in affected herds. To substantiate the above, we prospectively collected milk samples from herds with a *Staph. aureus* herd problem and compared them with those obtained from herds with sporadic *Staph. aureus* IMI. In addition, we analyzed inter- and intraherd variation of the staphylococcal VGP.

MATERIALS AND METHODS

Herd Selection

Based on the results of Fournier et al. (2008) and before starting the present study, we defined herds as *Staph. aureus* problem herds (case cohorts) if the quarter prevalence of *Staph. aureus* based on routine bacteriology (10-μL aliquot) was ≥10%. Control cohorts were defined as herds with a quarter prevalence of *Staph. aureus* from 0 to <10% (herds with sporadic IMI). The herds of both groups were spread over the western half of Switzerland (approximately 20,000 km^2). Cow breed and herd size were irrelevant. A total of 10 problem (265 cows) and 19 control herds (257 cows) were included in the present study.

To analyze whether the genotypes of *Staph. aureus* obtained from a particular infected quarter changed over time, 8 quarters (8 cows) with subclinical mastitis caused by *Staph. aureus* were sampled on d 1, 2, 4, and 7. Another series of 13 quarters (10 cows) was tested twice—at d 1 and between d 12 and 16. The corresponding herds were newly selected and were not associated with the problem or control herds mentioned above.

Sample Collection

In each of the 29 herds investigated, all lactating cows were checked for udder health, including visual milk inspection and clinical examination of the udder and teats, with the teat ends judged as described by Mein et al. (2001). Subsequently, milk of each quarter was aseptically collected for bacteriological testing and analysis of SCC, and the lactation number (**LN**) of each cow was recorded. The samples were transported and stored in the laboratory 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.

Staph. aureus Isolates

From the problem and control herds, 1,057 and 1,023 milk samples, respectively, were bacteriologically analyzed (10-μL aliquots) using standard procedures as described by the National Mastitis Council (NMC, 1999). In particular, *Staph. aureus* and other mastitis pathogens such as CNS or *Streptococcus* spp. were identified including colony morphology, biochemical properties, and detection of hemolysis. The problem herds provided 276 *Staph. aureus* isolates and the control herds yielded 25 isolates. All the isolates were further checked by PCR (see below) for the presence of the *nuc* gene, which codes for thermonuclease and is known to be specific for *Staph. aureus* (Brakstad et al., 1992; Graber et al., 2007). Isolates lacking this gene were excluded from the present study.

Extraction of Nucleic Acids

A single colony of *Staph. aureus* was resuspended in 100 μL of Tris-EDTA buffer (10 m*M* Tris/HCl, 1 m*M* EDTA, pH 8.0), incubated at 95°C for 10 min and placed immediately into ice. The resulting lysate was stored at −20°C until further use. For RS-PCR, the lysate was diluted 1:100 in H_2O and directly used for amplification. For all the remaining PCR, the lysate was purified: 30 μL of lysate was added to 170 μL of Tris-EDTA-sucrose buffer (100 m*M* Tris/HCl, 10 m*M* EDTA, 25% sucrose (wt/vol), pH 8.0) containing 0.5 mg of lysozyme (Merck, Berne, Switzerland) and 20 U of mutanolysin (Sigma, Buchs, Switzerland) for 60 min at 37°C. Total nucleic acid (**NA**) containing DNA and RNA was extracted using the High-Pure PCR Template Preparation Kit (Roche Diagnostics, Rotkreuz, Switzerland).

Primers

For *nuc* gene amplification, the primers of Graber et al. (2007) were applied, and for *coa* and *spa*, the primers of Akineden et al. (2001) were used. The SET genes *sea*, *seb-sec*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej* were amplified with the primers as described by Monday and Bohach (1999). For *tst*, we chose the primers according