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Bovine mastitis: The diagnostic properties of a PCR-based assay to monitor the *Staphylococcus aureus* genotype B status of a herd, using bulk tank milk

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ABSTRACT

Staphylococcus aureus genotype B (GTB) is a contagious mastitis pathogen in cattle, occurring in up to 87% of individuals. Because treatment is generally insufficient, culling is often required, leading to large economic loss in the Swiss dairy industry. As the detection of this pathogen in bulk tank milk (BTM) would greatly facilitate its control, a novel real-time quantitative PCR-based assay for BTM has previously been developed and is now being evaluated for its diagnostic properties at the herd level. Herds were initially classified as to their *Staph. aureus* GTB status by a reference method. Using BTM and herd pools of single-quarter and 4-quarter milk, the herds were then grouped by the novel assay, and the resulting classifications were compared. A total of 54 dairy herds were evaluated. Using the reference method, 21 herds were found to be GTB positive, whereas 33 were found to be negative. Considering the novel assay using both herd pools, all herds were grouped correctly, resulting in maximal diagnostic sensitivities (100%) and specificities (100%). For BTM samples, diagnostic sensitivities and specificities were 90 and 100%, respectively. Two herds were false negative in BTM, because cows with clinical signs of mastitis were not milked into the tank. Besides its excellent diagnostic properties, the assay is characterized by its low detection level, high efficiency, and its suitability for automation. Using the novel knowledge and assay, eradication of *Staph. aureus* GTB from a dairy herd may be considered as a realistic goal.

Key words: bulk tank milk, mastitis, polymerase chain reaction, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is worldwide one of the most important mastitis pathogens in cattle, involving large economic loss (Halasa et al., 2007; Hogeveen et al., 2011), mainly caused by chronic mastitis. Until now, *Staph. aureus* has largely been diagnosed and monitored by single-quarter milk samples using an aseptic sample technique and bacteriological culturing. However, this procedure is both laborious and time consuming. In addition, the cyclic shedding of *Staph. aureus* (Studer et al., 2008) and dead bacteria have a negative effect on the diagnostic sensitivity of conventional bacteriology (Graber et al., 2007). Although sensitivity is increased by repeated sampling, in individual cows it still remains between 41 and 100% (Sears et al., 1990) or even lower (Studer et al., 2008).

With the development of a ribosomal spacer PCR (**RS-PCR**) by Fournier et al. (2008), initially 17 *Staph. aureus* subtypes were isolated from milk samples of mastitic cows in Switzerland. Genotypes B (**GTB**) and C (**GTC**) were predominant; the other 15 genotypes were rare (Fournier et al., 2008). The same descriptive study as well as the prospective one of Graber et al. (2009) further revealed that *Staph. aureus* GTB is udder-associated and contagious, leading to herd problems, whereas *Staph. aureus* GTC and most of the other genotypes cause single-quarter infections, or are even nonpathogenic. *Staphylococcus aureus* GTB typically possesses the known enterotoxin genes *sea* and *sed*, as well as a polymorphism within the leucotoxin E gene (*lukE*, **lukEB**), as described by Fournier et al. (2008) and Graber et al. (2009). These genetic characteristics have been used to develop a novel analytical approach based on real-time quantitative PCR (**qPCR**), which is highly sensitive and specific for *Staph. aureus* GTB (Boss et al., 2011). All other genotypes, as well as the other mastitis pathogens, including *Staphylococcus* spp., could be unequivocally excluded (Boss et al., 2011). Moreover, the high sensitivity allows the assay to be applied to bulk tank milk (**BTM**), where the amount of *Staph. aureus* is often low. The calculated

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detection limit of the novel assay in BTM is 1 *Staph. aureus*-positive cow among 138 cows (Boss et al., 2011). Bulk tank milk was chosen because bulk milk sampling saves work and expense, and is easy to collect. In addition, BTM reflects the status of the herd, provided the milk of all lactating cows is delivered to the tank. Milk samples lacking this theoretical restriction are pools of milk obtained from each single cow of a herd, as used in the current study: 1) a herd pool of milk taken from all single quarters before milking (**QTRpool**) or 2) a herd pool of 4-quarter milk samples taken from each cow (**COWpool**) taken during milking.

The aim of the present field study was to evaluate the diagnostic properties of the GTB assay developed by Boss et al. (2011), at the herd level. The evaluation was performed according to the classical procedure, as recommended by the World Organisation for Animal Health (OIE, 2008). The dairy herds were first classified by the reference method (Fournier et al., 2008) into GTB-positive and GTB-negative herds. The same herds were then grouped by the novel assay of Boss et al. (2011), resulting in a proportion of herds that tested truly positive (= diagnostic sensitivity) and truly negative (= diagnostic specificity) in the assay, respectively. Because the different sample types (BTM, QTRpool, and COWpool) may influence the performance of the assay, the diagnostic properties were calculated for each type separately. In addition, SCC analysis was used to monitor the udder health and milk quality of the herds.

MATERIALS AND METHODS

Herd Selection

Fifty-four dairy farms with known GTB status comprising 1,150 cows were sampled between March 2010 and April 2011 in the western half of Switzerland. The GTB status of each herd was assessed using the reference method (RS-PCR) as developed by Fournier et al. (2008), which is described in more detail below.

The farms were typical of Switzerland, with respect to herd size (median = 16 cows). The median herd size of the present study was 18 lactating cows per farm, ranging from 10 to 57, as very small herds (<10 lactating cows) were excluded. The cows were kept in tiestall or freestall housing and milked twice per day. No restriction was placed on breed and farm management.

Milk Sampling

Each herd was sampled once, either during the morning or evening milking. Cows treated with antibiotics and cows up to 8 d postpartum were excluded according to legal requirements in Switzerland. For each herd, the following milk samples were collected: 1) single-quarter milk samples of each cow, taken under clean conditions before milking, and pooled for each herd by adding equal volumes (500 μL) in the same tube (QTRpool); 2) 4-quarter milk samples of each cow, taken during the milking process by continuously sampling an aliquot of milk from the claw of the cluster using a mechanical Tru-Test milk meter (Tru-Test Inc., Mineral Wells, TX), and pooled for each herd (COWpool); 3) 4-quarter milk samples of each cow containing the stabilizing agent Bronopol, destined for SCC analysis; and 4) BTM, taken after the whole milking process, following the guidelines of the National Mastitis Council (NMC, 1999), disregarding whether the bulk tank contained milk from 1 or multiple milking periods. All samples were transported at 4°C to the laboratory, immediately frozen, and stored at −20°C until further use. Samples for SCC analysis were stored at 4°C and processed within 1 d.

Reference Method

The reference method (Fournier et al., 2008) consists of 3 principal steps: 1) the cultivation of *Staph. aureus* from milk and DNA extraction; 2) the identification of the colonies by PCR; and 3) genotyping by RS-PCR.

Bacteria Cultivation and DNA Extraction. One hundred microliters QTRpool of each farm were plated on CHROMagar *Staph. aureus* plates (CHROMagar, Paris, France) and incubated at 37°C for 24 h. Ten mauve single colonies or as many as possible were then picked and each was inoculated in 100 μL of 10 m*M* Tris/HCl and 10 m*M* EDTA (pH = 8.5), incubated at 95°C for 10 min for lysis, and immediately placed on ice. These samples were diluted 1:100 in H_2O, so as to be used as a template for PCR with melting curve analysis (**mPCR**) and for genotyping by the reference method (see below).

nuc Gene mPCR. Detection of the thermonuclease gene (*nuc*) was performed by mPCR-analysis in a Rotor-Gene 6000 real-time thermal cycler (Corbett Life Science, Mortlake, Australia). The PCR reactions were run in a total volume of 20 μL, containing 1× Kapa Sybr Fast (Kapa Biosystems Inc., Woburn, MA) and 300 nmol of each primer (Table 1). Then, 2.5 μL of template was added to the reaction mix. The PCR steps were as follows: an initial step of 95°C for 3 min, 35 cycles of 95°C for 3 s and 60°C for 30 s, and a final elongation of 60°C for 5 min. Melting of amplicons was performed from 60 to 94°C, with rising steps of 1°C and a 5 s waiting time at each step. A single melting peak of 80.7 ± 1.96 × 0.14°C together with an appropriate melting curve of the positive control was regarded as a positive result for *Staph. aureus*.