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Mastitis diagnostics: Quantitative PCR for *Staphylococcus aureus* genotype B in bulk tank milk

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ABSTRACT

A novel real-time quantitative PCR assay for detecting the pathogenic and contagious *Staphylococcus aureus* genotype B (GTB) in bulk tank milk was developed and evaluated. The detection of this pathogen in bulk tank milk would greatly facilitate its control, as it is responsible for great economic loss in Swiss dairy herds. The assay is based on the simultaneous detection of 3 GTB-typical target sequences, including 2 enterotoxin genes and a polymorphism within the leucotoxin E gene. A variety of mastitis-associated bacteria was used to validate the assays, resulting in an analytical specificity of 100% and high repeatability. The analytical sensitivity in milk was 40 cfu/mL. An exponential association between simulated cow prevalence and quantitative PCR result was observed. An initial field study revealed 1 GTB-positive herd among the 33 studied herds. This novel assay for bulk tank milk analysis is suitable for routine purposes and is expected to be an effective tool for minimizing *Staph. aureus* GTB in Swiss dairy herds.
Key words: bulk tank milk, mastitis, polymerase chain reaction, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is the most common cause of contagious mastitis in cattle worldwide (Schällibaum, 1999; Zecconi et al., 2005). In Switzerland, it causes great economic loss (Kirchhofer et al., 2007); therefore, control of this pathogen would be beneficial. Unfortunately, conventional, routine bacteriology as a diagnostic tool is not entirely satisfactory, because the overall diagnostic sensitivity in single milk samples reaches just 79.9% (21.4 to 100%; Studer et al., 2008). Consequently, 3 consecutive samples are necessary to achieve a satisfactory diagnostic sensitivity (Sears et al., 1990), but it is often too expensive, so routine testing normally is accomplished with a single analysis. From a clinical point of view, this approach is not acceptable, as many cases of mastitis remain undetected, making the control of *Staph. aureus* mastitis difficult.

Graber et al. (2007) developed a highly sensitive and specific assay to detect *Staph. aureus* in raw milk samples. Its potential for automation in routine examinations is the basis for a wide range of use. This assay is >500 times more sensitive than conventional bacteriology and is highly specific for *Staph. aureus* (100%). It was evaluated in a longitudinal field study by Studer et al. (2008) and showed a diagnostic sensitivity of 99.4% and a diagnostic specificity of 97.1%.

In studies by Fournier et al. (2008) and Graber et al. (2009), various genotypes of *Staph. aureus* with different virulence and pathogenicity factors were identified and described. Genotype B (**GTB**) and genotype C (**GTC**) were predominant in Swiss dairy herds, whereas the remaining genotypes (**GTOG**) were rarely found. Genotype B was related to high contagiousness and increased pathogenicity, causing herd problems with cow prevalence up to 87% (Graber et al., 2009). By contrast, GTC and GTOG were found in infections of single cows.

Genotyping by Fournier et al. (2008) showed a high association between genotypes and virulence gene patterns. Among others, GTB was characterized by the presence of the *Staph. aureus* enterotoxin genes A (*sea*) and D (*sed*), and by a polymorphism within the leucotoxin E gene (*lukE*), caused by a point mutation, and called lukEB. Fournier et al. (2008) correctly identified 77% of GTB isolates by *sea*, 87% by *sed*, and 100% by lukEB. Correct negative results for non-GTB strains were observed in 100% of isolates for *sea*, 94% for *sed*, and 87% for lukEB.

As shown by Fournier et al. (2008) and Graber et al. (2009), *Staph. aureus* GTB infects many cows in a herd and requires infected herds to be sanitized to reduce SCC at herd level. From clinical experience, we know that proper sanitation alone takes 1 yr and is expensive because of treatment costs, loss of milk, culling, replacements, additional work, and veterinary support. To minimize IMI caused by *Staph. aureus* GTB at the herd

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and country levels, analysis of bulk tank milk (**BTM**) to detect and monitor GTB-positive herds would be the most efficient and economical method. Screening of BTM is a convenient, fast, and comparatively cheap method.

To improve the diagnostics for *Staph. aureus* as a mastitis pathogen, the goal of this study was to develop a novel assay to detect GTB in BTM. With the triple detection of lukEB, *sea*, and *sed* by real-time quantitative PCR (**QPCR**), this new method showed a high analytical sensitivity and specificity as well as a high repeatability.

MATERIALS AND METHODS

The development and validation of the assay specific for *Staph. aureus* GTB were undertaken according to guidelines proposed by the World Organization for Animal Health (OIE, 2008), adapted for QPCR. In particular, the analytical sensitivity and specificity of the assay as well as its repeatability were evaluated using a variety of bacterial DNA samples. The assay was then applied on simulated and real BTM samples.

Sequencing of the sea and sed Target Genes

In addition to lukEB, the genes *sea* and *sed* were chosen as QPCR targets because they have been shown to be typical for *Staph. aureus* GTB (Fournier et al., 2008; Graber et al., 2009). The DNA sequencing was performed to look for conserved regions of the *sea* and *sed* target genes. For both genes, 9 epidemiologically independent bovine strains of *Staph. aureus* were selected from our strain collection, and DNA was extracted from single colonies as described below. The unpurified lysates were diluted 1:100 in H_2O and were subjected to standard PCR for *sea* and *sed* (see below), respectively. The resulting amplicons were purified using the QIAquick PCR purification kit (Qiagen AG, Hombrechtikon, Switzerland).

The DNA concentration of the eluates was measured by spectroscopy using a Nanodrop ND-1000 spectrometer (Nanodrop Technologies, Rockland, DE); 100 ng was then added to a total volume of 10 μL containing 20 pmol of the *sea* or *sed* gene sequencing primers, respectively (Table 1). The sequencing procedures were done by the sequencing facility of Microsynth AG (Balgach, Switzerland).

Bacterial Strains

The DNA samples of udder pathogens with known identity were tested. Strains of *Staph. aureus* were obtained from former studies (Graber et al., 2007; Fournier et al., 2008) and initially isolated from bovine milk with spontaneous IMI. In total, there were 33 strains of *Staph. aureus* GTB, 18 strains of *Staph. aureus* GTC, and 20 strains of *Staph. aureus* GTOG, with the genotypes A, F, F′, G, I, J, K, L, M (Fournier et al., 2008), and newly detected genotypes J′, S, S′, and T resulting from the present study (see below). For CNS, 26 IMI-associated isolates were reused from the study of Graber et al. (2007) and 9 strains with known identity were obtained (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany): *Staph. hemolyticus* (DSM 20263), *Staph. warneri* (DSM 20316), *Staph. simulans* (DSM 20322), *Staph. intermedius* (DSM 20373), *Staph. chromogenes* (DSM 20454), *Staph. hyicus* (DSM 20459), *Staph. caprae* (DSM 20608), *Staph. xylosus* (DSM 20266), and *Staph. sciuri* ssp. *sciuri* (DSM 20345). Two strains each of the following pathogens were additionally included: *Streptococcus agalactiae*, *Strep. uberis*, *Strep. dysgalactiae* ssp. *dysgalactiae*, and *Enterococcus faecalis*, all identified by API 20 Strep (Biomérieux Suisse s.a., Geneva, Switzerland), 1 isolate of *Escherichia coli* identified by API 20 E, and 1 *E. coli* ATCC 8739 (American Type Culture Collection, Manassas, VA).

The bacteria were grown on 5% sheep blood agar plates (**BA**; Biomérieux Suisse s.a.) or brain heart agar plate (**BH**; Merck, Berne, Switzerland) for *Strep. uberis* and *Strep. dysgalactiae*. The plates were incubated at 37°C for 24 h.

DNA Extraction from Agar Plates and Purification

For extraction of bacterial nucleic acids (**NA**) containing both DNA and RNA, single colonies were picked from the BA or BH agar plates and inoculated in 100 μL of 10 m*M* Tris/HCl, 10 m*M* EDTA (pH = 8.5). The samples were incubated at 95°C for 10 min for lysis and immediately placed on ice.

To obtain purified DNA, 30 μL of the lysate was added to a mixture containing 150 μL of 25 m*M* Tris/HCl, 10 m*M* EDTA (pH = 7.2), 1.2×10^5 U of lysozyme (Merck), 8 μL of mutanolysine (2,000 U/mL; Sigma, Buchs, Switzerland), and 2 μL of DNase-free RNase (Roche Diagnostics AG, Rotkreuz, Switzerland). After incubation at 37°C for 30 min, 200 μL of binding buffer and 40 μL of proteinase K of the high pure PCR template preparation kit (Roche) were added and the samples processed and eluted in 100 μL of elution buffer as described by the manufacturer.

DNA concentrations were measured by spectroscopy (Nanodrop). The 260 nm:280 nm ratio had to be ≥1.80, otherwise the DNA extraction was repeated. Samples were diluted to 5×10^3, and 100 molecules/3.5 μL for *Staph. aureus* GTB samples or 5×10^5, 1.11×10^4