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

R. Boss,* J. Naskova,† A. Steiner,* and H. U. Graber*¹

*Clinic for Ruminants, Department of Clinical Veterinary Medicine, Vetsuisse-Faculty, University of Berne, 3001 Switzerland

†Agroscope Liebefeld-Posieux Research Station ALP, 3003 Berne, Switzerland

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.

Grabner 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 Grabner 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% (Grabner 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 Grabner 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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¹Corresponding author: hans.graber@knf.unibe.ch

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 H₂O 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 μ L 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 μ L of 10 mM Tris/HCl, 10 mM 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 μ L of the lysate was added to a mixture containing 150 μ L of 25 mM Tris/HCl, 10 mM EDTA (pH = 7.2), 1.2×10^5 U of lysozyme (Merck), 8 μ L of mutanolysine (2,000 U/mL; Sigma, Buchs, Switzerland), and 2 μ L of DNase-free RNase (Roche Diagnostics AG, Rotkreuz, Switzerland). After incubation at 37°C for 30 min, 200 μ L of binding buffer and 40 μ L of proteinase K of the high pure PCR template preparation kit (Roche) were added and the samples processed and eluted in 100 μ L 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 μ L for *Staph. aureus* GTB samples or 5×10^5 , 1.11×10^4

Table 1. Primers and probes used for real-time quantitative PCR (QPCR), standard PCR, and nucleotide sequencing

Gene ¹	Primer/probe	Sequence 5'–3'	Amplicon size (bp)
QPCR			
lukE	QlukEB-S	GGT GCT GAA GTA ATC AAA AGT ²	178
	QlukEB-AS	CAT ATC CAC TAC CCT TCA CAT C ²	
	QlukE-P	TG[A] GT[A] A[C]G [C]C[C] C[A]T ^{2,3}	168
	QseaM-S	GCC GAT CAA TTT ATG GCT AGA C ²	
sea	QseaM-AS	CTC TGA ACC TTC CCA TCA AAA AC ²	168
	QseaM-P	ACA GTA CCT TTG GAA ACG ^{2,4}	
sed		TTA CCG TAC AAG AAT	93
	QsedM-S	TAG ATG CAC AAG ²	
	QsedM-AS	GCT GTA TTT TTC CTC CGA GAG TAT CA ²	
	QsedM-P	AAG GCG CTA TTT GC ^{2,4}	
Standard PCR			
lukE	lukE-S	AAT GTT AGC TGC AAC TTT GTC ²	831
	lukE-AS	CTT TCT GCG TAA ATA CCA GTT CTA ²	
sea	Gsea-S	CTG AAT TGC AGG GAA CAG CTT TAG ²	512
	Gsea-AS	AAA CAC GAT TAA TCC CCT CTG AAC ²	
sed	Gsed-S	AAG TAG TAC CGC GCT AAA TAA TAT GA ²	624
	Gsed-AS	ATA TGA AGG TGC TCT GTG GAT AAT ²	
nuc	Nuc-S	CTG GCA TAT GTA TGG CAA TTG TT ⁵	664
	Nuc-AS	TAT TGA CCT GAA TCA GCG TTG TCT ⁵	
seg	Gseg-S	ACC TGA ACT ATC TAT AAG CGT GAA T ²	644
	Gseg-AS	TCT TTA GTG AGC CAG TGT CTT G ²	
sei	Gsei-S	GCG CAA GGA GAT ATT GGT GTA G ²	501
	Gsei-AS	AAC TTT CCC CTT ATT AAA ACC TG ²	
sej	Gsej-S	TAA CGA AAA GGG TAT CTC TGA A ²	332
	Gsej-AS	CTA TAT CTA GCG GAA CAA CAG T ²	
Nucleotide sequencing			
sea	GseaSeq-S	GGA ACA GCT TTA GGC AAT C ²	485
	GseaSeq-AS	CTC TGA ACC TTC CCA TCA A ²	
sed	GsedSeq-S	CGC GCT AAA TAA TAT GAA ACA TTC ²	525
	GsedSeq-AS	TAA GAG ACT TTA GAC CCA TCA GAA GA ²	

¹*lukE* = leucotoxin E gene; *sea* = *Staphylococcus aureus* enterotoxin gene A; *sed* = *Staph. aureus* enterotoxin gene D; *nuc* = thermonuclease gene; *seg* = *Staph. aureus* enterotoxin gene G; *sei* = *Staph. aureus* enterotoxin gene I; *sej* = *Staph. aureus* enterotoxin gene J.

²Reference: this paper.

³QPCR probe containing locked nucleic acids (in brackets) and labeled with 6-carboxyfluorescein (FAM) at the 5' end and with black hole quencher-1 at the 3' end.

⁴Minor groove binder QPCR probe labeled with FAM at the 5' end and with a confidential nonfluorescent quencher at the 3' end.

⁵Graber et al. (2007).

and 250 molecules/3.5 µL for DNA samples of all other germs. The sample volume used for QPCR was 3.5 µL.

Enterotoxin Gene Amplicons

Amplicons of *Staph. aureus* enterotoxin genes *sea*, *sed*, *seg*, *sei*, and *sej* were prepared by standard PCR (see below) using strains positive for these particular genes. The products were purified with the QIAquick PCR Purification Kit (Qiagen). Thereafter, the corresponding DNA concentrations were determined by spectroscopy (Nanodrop). Samples were diluted to 5×10^5 , 1.11×10^4 and 250 copies/3.5 µL. The sample volume used for QPCR was 3.5 µL.

Standard PCR and Gel Electrophoresis

For the amplification of the genes *sea*, *sed*, *seg*, *sei*, *sej*, thermonuclease (*nuc*), and *lukE*, standard PCR was performed in 25 µL containing 1× HotStarTaq Master Mix (Qiagen), 300 nM of each primer, and 2.5 µL of NA. All corresponding primers were synthesized by Microsynth and are listed in Table 1.

The PCR cycling included an initial step of 95°C for 15 min, followed by 35 cycles of denaturation at 94°C, annealing at 60°C, and extension at 72°C. Each step lasted 1 min, with a final elongation step at 72°C for 10 min. The samples were analyzed by agarose gel electrophoresis in 45 mM Tris-borate, 1 mM EDTA (pH = 8.3). Gel staining was done by adding 6 µL of

GelRed stock reagent (Biotium Inc., Hayward, CA) to 60 mL of hot gel solution. Bands were visualized using a standard UV transilluminator (312 nm).

Genotyping of *Staph. aureus*

Genotyping of *nuc*-positive isolates was performed as described by Fournier et al. (2008). In particular, the 16S to 23S rRNA intergenic spacer region was amplified by PCR (**RS-PCR**) using the primers of Jensen et al. (1993). The amplicons were then visualized with the miniaturized electrophoresis system, DNA 7500 Chip (Agilent Technologies, Basel, Switzerland). Newly found genotypes were named continuously according to Fournier et al. (2008).

Real-Time Quantitative PCR

Primers and probes for QPCR were designed with OLIGO 6 software (Molecular Biology Insights Inc., Cascade, CO) and are listed in Table 1. The probes for *sea* and *sed* were MGB labeled with 6-carboxyfluorescein at the 5' end and a confidential dark quencher at the 3' end synthesized by Applied Biosystems (Rotkreuz, Switzerland). All other primers and probes were synthesized by Microsynth. The probe for lukEB contained 6 locked nucleic acids at the positions indicated in Table 1. It was labeled with 6-carboxyfluorescein at the 5' end and with BHQ-1 at the 3' end. The sense primer QlukEB-S specifically detects the point mutation at nucleotide 132 of the *lukE* gene, typical for *Staph. aureus* GTB (Fournier et al., 2008; Graber et al., 2009). The primers and probes for *nuc*- and canine distemper virus gene N (**CDV-N**)-QPCR were the same as described by Graber et al. (2007).

The QPCR reactions were run in a total volume of 25 μ L containing 1 $\times$ Roche LightCycler 480 Probes Master (Roche). Considering QPCR for *nuc*, *sea*, and *sed*, the concentrations for both primers and the probe were 600 and 100 nM, respectively. For lukEB-QPCR, the concentrations were 300 and 200 nM, respectively, and for CDV-N-QPCR, the concentrations were 900 and 300 nM, respectively. In the case of bacteriological DNA samples obtained from agar plates and enterotoxin gene amplicons prepared by standard PCR, the QPCR reaction mix was additionally spiked with NA of 1.5×10^9 cfu of *Lactobacillus casei* to simulate extraction from milk. Finally, 3.5 μ L of template (NA, purified DNA, enterotoxin gene amplicons) was added to the QPCR mix.

The QPCR cycling started with an initial step of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 1 min. The QPCR was carried out in a Rotor-Gene 3000 real-time thermal cycler (Corbett Life Science, Mortlake, Australia).

The QPCR for all genes was run in duplicate. Results were considered positive if both reactions were positive. If only 1 reaction showed a positive result or the difference of the 2 cycle-to-threshold values was more than 2 cycles, the QPCR was repeated. A reaction was considered negative for all of the targets analyzed if amplification resulted in a value <10 copies/reaction. For each assay and each gene, a standard was included (in duplicates) ranging between 10 to 10^5 copies/assay. In the case of the *nuc* gene, 1 molecule was considered as 1 staphylococcal cell equivalent (Graber et al., 2007).

Assay Controls

Various controls were included to monitor the whole procedure. To evaluate the preparation of *Staph. aureus* from milk, 1 sample of sterile raw milk negative for lukEB, *sea*, *sed*, and *nuc*, as well as 1 raw milk sample spiked with 10^5 cfu/mL of *Staph. aureus* GTB positive for the 3 target sequences and *nuc*, were always coprocessed with the test samples. If the raw milk sample showed amplification or if the spiked sample was not within the adequate range ($\pm$ twice the expected non-transformed value), all test samples of the affected series were prepared again.

For each QPCR run, no-template controls were included. If the results were positive, the run was repeated.

To check for QPCR inhibitors present in NA, the samples were analyzed by an additional QPCR containing CDV-N gene amplicons as an internal control, which is detected by CDV-N-specific primers and a fluorescent probe (Graber et al., 2007). Negative QPCR results for the target sequences required a positive CDV-N QPCR result to exclude inhibitors.

Composite Cow Milk Samples

The 2,473 quarter milk samples of 629 dairy cows were reused from other studies (Moret-Stalder et al., 2009; Graber et al., 2009). The samples originated from 36 farms in the western half of Switzerland. Herds characterized by a quarter prevalence of *Staph. aureus* $\geq 10\%$ ($n = 11$) comprised 282 cows, whereof 127 were positive for GTB (Graber et al., 2009). Herds without *Staph. aureus* GTB-secreting cows ($n = 25$) comprised 347 cows. In these herds, the quarter prevalence of *Staph. aureus* was always <10% (Graber et al., 2009). The quarter milk samples had been collected aseptically using standard procedures (National Mastitis Council, 1999), transported on ice, and stored at -20°C . Breed and herd size were not considered.

For each cow, the corresponding quarter milk samples were pooled to create a composite cow milk (**CCM**)

sample by adding equal volumes of prewarmed (37°C) milk to a common tube.

From former studies, bacteriological results, SCC, and *Staph. aureus* genotypes obtained by RS-PCR (Fournier et al., 2008; Graber et al., 2009) were available.

Bulk Tank Milk Samples

An overview over all BTM samples is given in Table 2. The GTB-positive CCM samples ($n = 127$) were used to create (1) simulated BTM with a GTB cow prevalence of 5% (**p5BTM**; $n = 24$) and (2) simulated BTM with a GTB cow prevalence ranging from 18 to 95% (**pxBTM**; $n = 40$) based on the findings of Graber et al. (2009) in real herds. By assembling constant volumes of randomly attributed GTB-positive CCM samples (sampling procedure with putting back) and by adding a calculated volume of GTB-free milk, the attributed cow prevalence was achieved. To create simulated control herds (**cBTM**; $n = 25$), equal volumes of GTB-negative CCM were combined. For assembling, all milk samples were prewarmed to 37°C.

Real BTM (**rBTM**) was collected from 33 herds (12 to 58 cows per herd) in the area of Berne, Switzerland, all of which were attended by the same practicing veterinarian. Without any knowledge about cow udder health status, the first 33 farmers who agreed were visited. After stirring the milk tank for 10 min, BTM was collected according to NMC (1999) guidelines, transported on ice, and stored at -20°C until further use.

Preparation of bacteria from milk using *L. casei* and NA extraction were performed according to Graber et al. (2007) with the following modification: for cell lysis, the pelleted bacteria were resuspended in 150 μ L of 25 mM Tris/HCl, 10 mM EDTA (pH = 7.2), incubated at 95°C for 10 min, and immediately placed on ice. The final elution volume was 200 μ L.

Bacteriology

Conventional bacteriology from rBTM samples was performed as proposed by the NMC (1999). In particular, the following agar plates were used: BA for a general overview and total bacterial growth, modified Edwards agar (**ED**; Oxoid, Basel, Switzerland) for the detection of streptococci and related organisms, Vogel-Johnson agar (**VJ**; Becton Dickinson AG, Basel, Switzerland) for the identification of staphylococci, and MacConkey agar (**MC**; Oxoid) for the detection of *Enterobacteriaceae*, including coliforms. The prewarmed (37°C) rBTM samples were intensively vortexed, and 10 μ L of each sample was then spread over each of the different agar plates using sterile triangle spatulas. After incubation for 24 h and 48 h, respectively, colonies were

Table 2. List of all bulk tank milk (BTM) samples as an overview

BTM sample ¹	n	Cow prevalence of GTB ²	Herd size (cows)
cBTM	25	0	4–44
p5BTM	24	5	20
pxBTM	40	18–95	11–60
rBTM	33	Unknown	12–58

¹cBTM = control herds (simulated); p5BTM = simulated problem herds with 5% cow prevalence; pxBTM = simulated problem herds with different cow prevalences; rBTM = real BTM.

²GTB = *Staphylococcus aureus* genotype B.

counted as proposed by the NMC (1999). For the final results, growth after 48 h was considered and noted as semiquantitative values: –, no growth, 0 colonies; +, weak growth, 1 to 10 colonies; ++, medium growth, 10 to 100 colonies; +++, intense growth, >100 colonies.

Additionally, Chromagar *Staph. aureus* plates (CHROMagar, Paris, France) specific for *Staph. aureus* were inoculated with 100 μ L of 11 selected rBTM samples to search for other genotypes. After incubation at 37°C for 24 h, 10 mauve colonies per plate were subcultured on BA. One colony of each BA plate was then picked and checked for *Staph. aureus* using *nuc* gene PCR (see above), and all positives were genotyped by RS-PCR as described above.

Statistical Analysis

Data are expressed as absolute numbers, percentages, or as log-transformed values (logarithm to the basis 10). Robust nonlinear regression analysis was performed, with the QPCR results [$\log_{10}(\text{copies/reaction})$] forming the independent variable, and the simulated cow prevalence of a herd (%) acting as the dependent variable. The model was selected that produced the maximal coefficient of determination (R^2), resulting in an exponential dependency between both variables. For all of the statistical analyses, the Systat 12 software package (Systat Software Inc., Richmond, CA) was used. Values of $P < 0.05$ were considered significant.

RESULTS

Target Gene Analysis

To obtain a solid basis for selecting primers that allow for consistent *sea* and *sed* gene amplifications of bovine strains, 9 epidemiologically unrelated *Staph. aureus* isolates obtained from bovine mastitic milk were sequenced for both genes.

For the *sea* gene, all 9 strains showed a 100% identity within the 448 sequenced nucleotides. In addition, they were 100% identical to the reference *sea* sequence (GenBank BX571857).

Table 3. Examined DNA samples and their real-time quantitative PCR (QPCR) results for the 3 targets and the resulting analytical specificities

DNA sample ¹	n	lukEB ²		sea ²		sed ²	
		Neg. ³	Pos. ³	Neg.	Pos.	Neg.	Pos.
GTB	33	0	33	3	30	0	33
Analytical specificity (%)		0	100	9	91	0	100
Non-GTB							
GTC	18	18	0	18	0	18	0
GTOG	20	14	6	20	0	19	1
CNS	35	35	0	35	0	35	0
Other bacteria	10	10	0	10	0	10	0
Enterotoxin genes	4	ND ⁴	ND	4	0	4	0
Total non-GTB		77	6	87	0	86	1
Analytical specificity (%)		93	7	100	0	99	1

¹GTB = *Staphylococcus aureus* genotype B; GTC = *Staph. aureus* genotype C; GTOG = other genotypes than GTB and GTC.

²Target genes: lukEB = point mutation within leucotoxin E gene, typical of GTB; sea = *Staph. aureus* enterotoxin gene A; sed = *Staph. aureus* enterotoxin gene D.

³Neg. = number of negative QPCR results; Pos. = number of positive QPCR results.

⁴ND = not determined.

Considering the *sed* gene, comparison of the 9 bovine sequences (each 560 nucleotides long) to the reference *sed* sequence (GenBank M28521) resulted in similarities ranging from 98.4 to 99.6%. All 9 strains showed a transition at nucleotide 674, resulting in a substitution of Arg to Lys and a transversion at nucleotide 631, leading to an exchange of Pro against Ala. Two of the 9 strains showed further transitions at nucleotides 499 (Asp to Asn), 674 (Arg to Lys), 932 (Ser to Phe), 998 (Arg to Gln), and 1005 (silent mutation), and transversions at nucleotides 631 (Pro to Ala), 667 (Ile to Lys), 812 (Asn to Ile), and 860 (Lys to Thr). Two other strains further showed a deletion at nucleotide 805, resulting in a chain termination 6 AA downstream.

Based on these findings, 2 primers and a fluorescent probe for QPCR were designed for both genes (Table 1). They were expected to detect the *sea* and *sed* genes of all the bovine and human *Staph. aureus* isolates specifically.

Specific amplification also was expected for the GTB-typical polymorphism of *lukE* (point mutation). As based on previous studies using restriction-length polymorphism and DNA sequencing for differentiation, the point mutation could be detected by the lukEB-QPCR sense primer designed for this study (Table 1).

Real-Time Quantitative Assay Performance

Using serial dilutions of purified lukEB, *sea* and *sed* amplicons, the assays were shown to give log-linear results between 10 and 10⁵ copies/assay. For this range, the coefficient of correlation of each of the 3 QPCR was 0.999. The amplification efficiency was 1.02 for lukEB and *sed*, and 1.01 for *sea* and *nuc*, as measured on the gradient.

Analytical Specificity of the Single-Target QPCR

For lukEB-QPCR, the analytical specificity was 93% (77/83; Table 3). Six out of 20 *Staph. aureus* GTOG strains tested positive for lukEB. All of the *Staph. aureus* GTC, CNS, and other bacteria were lukEB-QPCR negative. In addition, all of the *Staph. aureus* GTB strains tested positive for lukEB.

For *sea*-QPCR, the analytical specificity was 100% (87/87; Table 3). All of the *Staph. aureus* GTC, GTOG, CNS, and other bacteria, and all of the other enterotoxin genes were *sea*-QPCR negative. In addition, 30 out of 33 *Staph. aureus* GTB strains tested positive for *sea* (91%).

For *sed*-QPCR, the analytical specificity was 99% (86/87; Table 3). One out of 20 *Staph. aureus* GTOG strains tested positive for *sed*. All of the *Staph. aureus* GTC, CNS, and other bacteria, and all of the other enterotoxin genes were *sed*-QPCR negative. In addition, all of the *Staph. aureus* GTB strains tested positive for *sed*.

Analytical Specificity of the Assay

Using the analytical specificity of the single targets (Table 3), the probabilities of observing different combinations of QPCR results were calculated (Table 4) given that a milk sample was truly *Staph. aureus* GTB positive or negative, respectively. For a sample positive for the 3 targets (lukEB+, *sea*+, *sed*+), the probability of being GTB positive was 0.909 ($1 \times 0.909 \times 1$); the probability of being a non-GTB was 0 ($0.072 \times 0 \times 0.012$). For lukEB+, *sea*-, and *sed*+ samples, the probabilities were 0.091 and 0.001, respectively. All of the other combinations were typical of GTB-negative samples or were improbable (Table 4).

Table 4. Discrimination: probabilities of milk samples containing *Staphylococcus aureus* genotype B (GTB) or non-GTB, combining the real-time quantitative PCR (QPCR) results for the 3 targets

Combination of QPCR results ¹			Probabilities	
lukEB	<i>sea</i>	<i>sed</i>	GTB	Non-GTB
+	+	+	0.909	0
+	–	+	0.091	0.001
+	+	–	0	0
+	–	–	0	0.071
–	+	+	0	0
–	–	+	0	0.011
–	+	–	0	0
–	–	–	0	0.918

¹lukEB = point mutation within leucotoxin E gene, typical of GTB; *sea* = *Staph. aureus* enterotoxin gene A; *sed* = *Staph. aureus* enterotoxin gene D; + (positive) = amplification over limit of detection; – (negative) = no amplification.

Analytical Sensitivity

The analytical sensitivity of the different QPCR assays was evaluated by serial 1:10 dilutions of milk spiked initially with 10^5 cfu/mL of *Staph. aureus* GTB positive for the 3 target sequences. The limits of detection were 40 cfu/mL milk for lukEB, 100 cfu/mL for *sea*, and 10 cfu/mL for *sed*.

Repeatability

The intraassay variability was determined by simultaneous extraction ($n = 8$) of the same milk sample spiked with 10^5 cfu/mL of *Staph. aureus* GTB. The samples were completely analyzed (bacteria preparation, NA extraction, QPCR) 8 times for the targets (i.e., lukEB, *sea*, and *sed*). The coefficient of variation was 2.9% for lukEB, 0.9% for *sea*, and 1.6% for *sed* ($\log_{10}$ -transformed values). The interassay variability was evaluated by a complete analysis of milk samples ($n = 10$) spiked with 10^5 cfu/mL of *Staph. aureus* GTB on 10 different days. The coefficient of variation was 1.7% for lukEB, 2.1% for *sea*, and 1.8% for *sed* ($\log_{10}$ -transformed values).

Relationship Between Gene Copy Numbers and Colony Forming Units

To compare the number of the copies of *sea*, *sed*, and lukEB genes to the copy number of the *nuc* gene, which is a true estimation of the number of *Staph. aureus* cells, a GTB strain was cultured in tryptic soy broth (Becton Dickinson) at 37°C overnight. The DNA extraction and QPCR for the corresponding genes were performed as described above. Compared with the *nuc* gene, an approximate 4-fold and an approximate 3- to 4-fold increase was observed for lukEB and *sed*, respectively. In case of *sea*, the ratio was approximately 1.

Bulk Tank Milk Samples

Overall, an exponential dependency ($R^2 = 0.861$; $P < 0.001$) was observed between GTB cow prevalence of a herd and the logarithmic lukEB-QPCR value (number of copies per reaction) measured in simulated BTM samples (Figure 1). All cBTM samples ($n = 25$) were tested negative for lukEB and appear as a single point in the intersection of the axes (Figure 1). All p5BTM samples ($n = 23$) were tested positive for lukEB and were located within a wide range varying from 69 to 7.67×10^3 copies/reaction. One value was an extreme outlier (6.6×10^4 copies/reaction) and was therefore not considered for further analyses. All pxBTM samples ($n = 40$) tested positive for lukEB and ranged from 2.61×10^3 to 7.93×10^5 copies/reaction.

Thirty-three rBTM samples were examined by QPCR for the 3 targets, as well as for *nuc* (Table 5). One rBTM sample (3%) was identified as positive for *Staph. aureus* GTB because of its positive QPCR results for all targets (i.e., lukEB, *sea*, and *sed*). Twenty rBTM samples (61%) were negative for all targets. The 12 remaining rBTM samples (36%) were positive for 1 or 2 targets.

All rBTM samples positive for lukEB- and *nuc*-QPCR ($n = 11$) were furthermore reverse examined; for each sample, 10 mauve single colonies were selected from Chromagar and subcultured on BA followed by genotyping all those colonies that were found to be *nuc* positive by standard PCR ($n = 72$). In total, 8 different genotypes were found (i.e., A, B, C, and F, and the newly found genotypes J', S, S', and T; Table 5). Considering farm No. 17, all isolates were confirmed as GTB by genotyping. In 2 rBTM samples, more than 1 genotype was found, and in other 2 samples, none of the colonies was positive for *nuc* (Table 5).

Bacteriological examination using 10 μ L of milk revealed mid-range to intense growth on BA for all 33

Figure 1. *Staphylococcus aureus* genotype B (GTB): correlation between cow prevalence in simulated bulk tank milk samples and the corresponding real-time quantitative PCR results ($\log_{10}$ -transformed) for lukEB. Robust nonlinear analysis resulted in $R^2 = 0.861$ ($P < 0.001$). lukEB = point mutation within leucotoxin E gene, typical of GTB; ■ control herds (cBTM; $n = 25$; no GTB secreting cows); ● problem herds (p5BTM; $n = 23$; cow prevalence of 5%); ▲ problem herds (pxBTM; $n = 40$; percentage of GTB secreting cows ranging from 18 to 95%).

rBTM samples. In the majority of the samples, *Streptococcus* spp. (79%) and *Staphylococcus* spp. (88%) were observed. *Enterobacteriaceae* were found in 18% of the samples. Only 8/29 (28%) samples with growth on VJ were positive for *nuc* QPCR, but all *nuc*-positive samples showed staphylococcal growth on VJ agar.

The BTM of the GTB-positive farm (No. 17) showed intense growth on BA, medium growth on VJ, weak growth on ED, and no growth on MC agar.

DISCUSSION

The present study describes a novel QPCR-based assay to detect *Staph. aureus* GTB in BTM. The assay is highly sensitive and specific for this contagious mastitis pathogen and is characterized by a small intra- and interassay variability. It includes preparation of bacteria from milk as described by Graber et al. (2007) and QPCR for the 3 GTB-typical targets lukEB, *sea*, and *sed* (Fournier et al., 2008; Graber et al., 2009). A BTM sample is GTB-positive if QPCR for lukEB and *sed* and/or *sea* generates a positive result each ≥ 10 copies/reaction. Quantification is based on the lukEB target. The BTM was used as it is the most efficient

and economical way of detecting and monitoring GTB-positive herds.

So far, BTM analyses for *Staph. aureus* using conventional bacteriological examination were of limited value as only a weak association between cfu of *Staph. aureus* measured in BTM and the number of infected cows has been observed (Gonzalez et al., 1986; Farnsworth, 1993). This observation is not surprising as *Staph. aureus* in BTM also may originate from sites other than the mammary gland (environmental *Staph. aureus*), including the teat and udder skin, parlor, milking machine, air, and bedding (reviewed by Sears and McCarthy, 2003). According to Fox et al. (1991) and Zadoks et al. (2002), however, *Staph. aureus* associated with IMI differed from the environmental ones as shown by pulsed-field gel electrophoresis or phage typing and may therefore be considered mastitis irrelevant. To complicate matters further, some IMI-associated subtypes are not pathogenic (Fournier et al., 2008). The presented QPCR analytics, however, allow, for the first time, the highly sensitive detection of an actual IMI-associated and pathogenic subtype of *Staph. aureus* (GTB) in BTM, to the exclusion of all remaining and interfering subtypes.

Considering the analytical specificity of the assay, 100% of the GTB-positive strains ($n = 33$) were correctly detected. In addition, all remaining genotypes ($n = 38$), CNS ($n = 35$), other mastitis-causing and environmental bacteria ($n = 10$), as well as further enterotoxin genes (*sei*, *sej*, *seg*), were safely excluded. Specificity remained the same when low and high concentrations of non-GTB targets were used. Furthermore, QPCR permitted detection of targets within a wide dynamic range, rendering dilution steps of samples unnecessary.

The high analytical specificity of the present GTB-QPCR analytics was accompanied by a high analytical sensitivity as the detection limit for lukEB was 40 cfu of *Staph. aureus* GTB per milliliter of milk. In addition, the repeatability of the lukEB-, *sea*-, and *sed*-QPCR assays were high. Based on the $\log_{10}$ -transformed values, the intraassay variability of the 3 QPCR ranged from 0.9 to 2.9%, the interassay variability ranged from 1.7 to 2.1%. False-negative QPCR results caused by competitive or enzymatic inhibitors present in the sample NA were ruled out by inclusion and detection of an internal control (CDV-N gene). Multiplexing the 3 different QPCR was never considered. The main reasons for this decision were the reduced analytical sensitivity and efficiency of these QPCR assays, as well as the occurrence of false-negative results because of competing reactions (Henegariu et al., 1997).

To get an impression of how the novel methodology will perform in future extended field studies, simulation analyses (Figure 1) and an initial, exploratory field

Table 5. Real bulk tank milk samples (n = 33): real-time quantitative PCR (QPCR) results for all detected targets, genotype (ribosomal spacer PCR), and semiquantitative bacteriological findings after a 48-h incubation (10 µL inoculated)

No.	QPCR targets ¹				Genotype ²	Bacteriology ³			
	<i>nuc</i>	lukEB	<i>sea</i>	<i>sed</i>		BA	ED	VJ	MC
1	Pos.	–	–	Pos.	C	++	–	+	–
2	–	–	–	–		++	–	+	–
3	–	Pos.	–	–	C	+++	++	++	+
4	–	–	–	–		++	+	++	–
5	–	–	–	–		++	+	–	–
6	–	–	–	–		+++	+	++	–
7	Pos.	–	–	–	C	++	+	+	–
8	–	–	–	–		++	+	+	+
9	–	–	–	–		++	+	+	–
10	–	–	–	–		++	+	–	–
11	–	–	–	–		++	+	++	–
12	Pos.	–	–	–	F, S, S'	++	+	+	–
13	–	–	–	–		++	+	++	–
14	–	–	–	–		++	+	+	+
15	–	–	–	–		++	–	+	–
16	–	–	–	–		++	–	+	–
17	Pos.	Pos.	Pos.	Pos.	B	+++	+	++	–
18	Pos.	–	–	–	A	+++	++	++	–
19	–	–	–	–		++	+	+	+
20	Pos.	–	–	–	T	++	+	++	–
21	–	–	–	–		+++	+	+	–
22	–	Pos.	–	–	None ²	+++	+++	+	+
23	–	–	–	–		++	–	+	–
24	–	–	–	–		++	–	–	–
25	–	Pos.	–	–	None	++	+	+	–
26	–	–	–	Pos.		++	+	+	–
27	–	–	–	–		++	+	+	–
28	–	–	–	–		++	+	+	–
29	Pos.	–	–	–	C	++	++	+	++
30	–	–	–	–		++	–	+	–
31	–	–	–	–		+++	+	++	–
32	Pos.	–	–	–	C, J'	+++	++	++	–
33	–	–	–	Pos.		++	+	–	–

¹*nuc* = thermonuclease gene; lukEB = point mutation within leucotoxin E gene, typical of GTB; *sea* = *Staphylococcus aureus* enterotoxin gene A; *sed* = *Staph. aureus* enterotoxin gene D; Pos. (positive) = amplification over limit of detection; – (negative) = no amplification.

²None = no *Staph. aureus* colonies isolated.

³BA = blood agar; ED = Edwards agar; VJ = Vogel-Johnson agar; MC = MacConkey agar; –, no growth (0 colony); +, weak growth (1–10 colonies); ++, medium growth (10–100 colonies); +++, intense growth (>100 colonies).

study were performed. An exponential dependency ($R^2 = 0.861$; $P < 0.001$) between the logarithmic lukEB value and the simulated GTB cow prevalence was found. These investigations further revealed that the lowest p5BTM value accounted for 69 lukEB copies/reaction. As the detection limit of the lukEB-QPCR is 10 copies/reaction, the minimal theoretical cow prevalence to be detected is 0.72% ($5 \times 10/69$), which translates to an expected detection rate of about 1 lukEB-positive cow among 138 cows using BTM. The p5BTM data also demonstrate the large range of lukEB values, resulting from single cow infections. If more cows are infected, pooling of milk averages the different amount of *Staph. aureus*, so that extreme values become impossible. Indeed, as shown in Figure 1, outliers were not observed when the cow prevalence was above 20%.

Bacteriological analysis of the rBTM samples showed mid-range to intense growth on BA and limited growth of streptococci and related bacteria (ED agar). Only 18% of the samples were MC positive, which is an indicator of fecal contamination (coliforms). These results demonstrate that milking was properly carried out in most cases and that standards of hygiene were within an acceptable range. Considering VJ agar (NMC, 1999), staphylococci were observed in 29 of the 33 rBTM samples (88%). Compared with *nuc* QPCR (Table 5), which is highly sensitive and specific for *Staph. aureus* (Graber et al., 2007), VJ agar generated many more positive results, suggesting that at least some of them are false positive. These results are not surprising as a considerable number of CNS strains ferment mannitol and reduce tellurite (Holt et al., 1986).

The subject of the present study was the development and technical evaluation of a novel methodology to detect *Staph. aureus* GTB in milk and to get an impression by simulation analyses and an initial field study of how this technology will perform in reality using BTM. Future extended field studies are necessary; in particular, investigations need to be performed focusing on the diagnostic and practical performance of the analytics as well as on the financial effect at the herd and country level when the novel methodology will be applied together with an appropriate control strategy.

CONCLUSIONS

The described QPCR analytics is a fast and potent method of screening easily available BTM samples for the *Staph. aureus* GTB. It is characterized by high analytical specificity and sensitivity as well as by high repeatability. Based on our simulation experiments and an initial field study, the methodology is expected to provide a powerful tool for the control of this contagious pathogen in the future.

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