Development of a new real-time quantitative PCR assay for the detection of *Staphylococcus aureus* genotype B in cow milk, targeting the new gene *adlb*

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ABSTRACT

The specific and reliable diagnosis of mastitis pathogens is essential for successful sanitation programs. The aim of the present study was to develop and evaluate a new real-time quantitative PCR (qPCR) assay for the very sensitive and specific detection of *Staphylococcus aureus* genotype B in cow milk samples. This mastitis pathogen is contagious and particularly prevalent in Switzerland and other central European countries. The new test is based on a rapid preparation of bacteria, followed by DNA isolation and qPCR for a unique target gene coding for the adhesion-like bovine protein (*adlb*). The inclusivity of the new target gene was 97% and the exclusivity 98%, meaning that other genotypes and bacterial species could be excluded with high reliability. The limit of detection of the new assay was 235 staphylococcal cell equivalents/mL of culture. The new test shows high intra- and interassay repeatability. Results are available within 2 d after sampling, allowing farmers and veterinarians to apply sanitation measures immediately. Based on the results of a preliminary field study, the diagnostic sensitivity and specificity of the new qPCR assay are 99 and 100%, respectively. The new analytical procedure is straightforward and can be applied for routine diagnostics.

Key words: mastitis, milk, *Staphylococcus aureus*, diagnostics, real-time quantitative PCR

INTRODUCTION

Staphylococcus aureus is one of the most prevalent pathogens causing IMI in cattle, responsible for substantial milk, quality, and economic loss in dairy farming worldwide (Barkema et al., 2009; Halasa et al., 2009; Hogeveen et al., 2011; Fluit, 2012). This pathogen typically causes subclinical, chronic mastitis (Sears

and McCarthy, 2003; Petersson-Wolfe et al., 2010), and previous studies confirmed that various genotypes of *Staph. aureus* exist, differing in their infection behavior (Sommerhäuser et al., 2003; Fournier et al., 2008; Graber et al., 2009). Important pathogenicity factors, making the therapy and elimination of *Staph. aureus* much more difficult, include the ability of the bacterium to persist intracellularly within mammary epithelial cells and neutrophils (Löffler et al., 2014; Peton and Le Loir, 2014), to evade the innate immune response of infected hosts (Zecconi and Scali, 2013), and to build microabscesses (Fox et al., 2001). Additionally, *Staph. aureus* can build biofilms, which are clusters of bacteria embedded in polysaccharides, proteins, and extracellular DNA (Fluit, 2012). Biofilm-forming bacteria are more resistant against the host immune response and antibiotic therapy (Fluit 2012; Peton and Le Loir, 2014). Finally, *Staph. aureus* represents a potential public health threat because of its ability to produce enterotoxins (Peton and Le Loir, 2014). Enterotoxins can lead to staphylococcal food poisoning, characterized by abdominal pain, vomiting, and diarrhea appearing 0.5 to 8 h after ingestion of contaminated food (Zhang and Stewart, 2001).

Control and eradication (sanitation) programs represent the most promising strategy for farmers to prevent contagious mastitis (Petersson-Wolfe et al., 2010). Implementation of such programs is reasonable because of the often unsatisfactory cure rate, which can be achieved with antibiotic therapy against *Staph. aureus* in single cows (Sol et al., 1997; Sol et al., 2000; Gruet et al., 2001; Sears and McCarthy, 2003; Barkema et al., 2006). Further reasons are the inappropriate use of antibiotics for mastitis therapy (Pyörälä, 2009), as well as the limited efficacy of vaccination against *Staph. aureus* IMI (Peton and Le Loir, 2014; Schukken et al., 2014; Landin et al., 2015).

The analytical method to detect *Staph. aureus* in mastitis milk samples is fundamental. Classical, phenotypical bacteriology currently represents the gold-standard for routine analysis of clinical milk samples (Koskinen

Received March 2, 2017.

Accepted June 2, 2017.

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et al., 2010), probably because most diagnostics laboratories offer this kind of analysis and because costs are relatively low compared with more advanced molecular biology methods. Bacteriological culturing for *Staph. aureus* has some important limitations, such as the time required for analysis, the objective interpretation of results, and the nonsatisfactory analytical sensitivity and specificity (Studer et al., 2008; Koskinen et al., 2009; Hogeveen et al., 2011). Furthermore, as bacteriology is a phenotypical method, it does not differ between genotypes. Finally, 3 consecutive analyses of aseptically collected milk samples are required to reach a high diagnostic sensitivity (Sears et al., 1990; Studer et al., 2008). Therefore, the minimum time required to clarify the status of an animal is about 1 mo, considering time intervals of 2 to 3 wk between samplings (Kirchhofer et al., 2011). This extended time interval represents an elevated risk for new infections of herd mates.

For *Staph. aureus*, the possibility to specifically discriminate between the different genotypes is crucial, because they are characterized by different epidemiological, clinical, and pathogenicity properties (Fournier et al., 2008), and only genotype B (**GTB**) exhibits a contagious model of transmission (Fournier et al., 2008; Cremonesi et al., 2015; van den Borne et al., 2017). *Staphylococcus aureus* GTB is characterized by high prevalence, both at the quarter and at the cow level, whereas genotype C (**GTC**) and other genotypes (**GTOG**) are responsible for single-quarter, sporadic infections of individual cows (Fournier et al., 2008; Graber et al., 2009). According to Fournier et al. (2008), *Staph. aureus* GTB shows a typical virulence gene pattern, including the presence of the enterotoxin genes *sea*, *sed*, and *sej*, and a SNP within the leukotoxin E (*lukE*) gene called lukEB. This specific pattern provides scope for the differentiation of GTB from other genotypes. Finally, GTB shows an increased pathogenicity compared with GTC and GTOG, which is reflected in higher SCC in the milk of infected cows (Fournier et al., 2008).

As previously described (Cosandey et al., 2016), about 10% of the Swiss dairy herds are positive for *Staph. aureus* GTB. This estimation was based on the analysis of 223 bulk tank milk samples stratified among the number of dairy herds per canton; the same analyses further showed local differences among the herd prevalence for *Staph. aureus* GTB (Cosandey et al., 2016). As described by Voelk et al. (2014) and van den Borne et al. (2017), in mountainous regions of Switzerland cows from several different herds are typically brought together during the summer months on communal operations (alps), where they are pastured and milked together, sharing the milking equipment and housing facilities. The study by Voelk et al. (2014),

investigating the prevalence of *Staph. aureus* GTB on alps, revealed that the within-herd GTB prevalence reached up to 72% of the cows at the end of the summer season. The milk produced on alps is mostly made into raw milk cheese, and the study by Hummerjohann et al. (2014) showed that *Staph. aureus* GTB is the most abundant subtype of *Staph. aureus* isolated from these cheeses. The successful transfer of *Staph. aureus* GTB from the bovine udder to the cheese production chain was further observed in a recent study conducted in Austria by Kuemmel et al. (2016), confirming the importance of this udder pathogen for food safety. According to Heiniger et al. (2014), *Staph. aureus* GTB is responsible for about 60% of the total mastitis cost in Switzerland, corresponding to approximately 80 million Swiss Francs per year.

Considering the notable prevalence of *Staph. aureus* GTB in Switzerland, as well as its implications for udder health, food safety, and its economic impact on Swiss agriculture, the aim of the present study was to develop and evaluate a simplified real-time quantitative (q)PCR assay for the very specific and sensitive detection of *Staph. aureus* GTB in bovine milk samples, targeting the new, single copy gene *adlb*, which codes for the adhesion-like bovine protein. The new test represents a very solid diagnostic tool to routinely monitor the *Staph. aureus* GTB status of dairy herds, as well as to support the decision-making process during sanitation programs for this contagious udder pathogen.

MATERIALS AND METHODS

The development and validation of the new qPCR assay for *Staph. aureus* GTB in bovine milk samples are based on the guidelines proposed by the World Organisation for Animal Health (OIE, 2016a,b,c). All primers and probes used for the development of the new qPCR assay (including those for standard PCR and for sequencing of the target gene) were designed using the Oligo 6.51 software (Molecular Biology Insights Inc., Cascade, CO), synthesized by Microsynth (Balgach, Switzerland) or Thermo Fisher Scientific (Darmstadt, Germany), and are listed in Table 1.

Selection of Target Gene, Primers, and Probe for qPCR

To find a unique target gene specific for *Staph. aureus* GTB, 2 corresponding strains (M5512B, M6020B) isolated from subclinical bovine IMI were sequenced by next-generation sequencing using 454 technology (Roche, Basel, Switzerland). This procedure including the assembly of the contigs was performed by Microsynth. The obtained contigs were aligned to *Staph. au-*

reus NCTC8325 using Mauve 2.3.1. software (Darling et al., 2004) and joined to construct a full chromosomal genome. Using Clone Manager 9.1 software (Scientific & Educational Software, Denver, CO) and multiple sequence alignments, the GTB genomes were compared with complete *Staph. aureus* genomes present in the Nucleotide database (National Center for Biotechnology Information, Bethesda, MD), including N315, MW2, USA300, MSSA176, MRSA252 (all human strains), RF122 (bovine), ED133 (sheep), and ED98 (poultry). Based on these analyses, a GTB-specific target gene was detected. Appropriate primers and a hydrolysis probe were then designed (Table 1) using Oligo 6.51 software (Molecular Biology Insights). The probe contained a confidential minor groove binder and was labeled with 6-carboxyfluorescein (FAM) at the 5' end and with a confidential quencher at the 3' end (Thermo Fisher Scientific). The novel target gene was further characterized in silico using Interpro (EMBL-EBI, Hinxton, UK), Uniprot (The UniProt Consortium,

Geneva, Switzerland), and Clone Manager 9.1 Software (Scientific & Educational Software).

Standard PCR and DNA Sequencing of the Target Gene

Sequencing was carried out to look for conserved regions within the target gene used later on to develop the new qPCR assay. The aim was to reduce the risk of false-negative and false-positive results for the definite assay. For this purpose, 25 *Staph. aureus* GTB strains from our strain collection were randomly selected and cultured on blood agar (BA) medium (Biomérieux Suisse s.a., Geneva, Switzerland) at 37°C overnight. The DNA was extracted by boiling (see below). For the amplification of the *adlb* gene, standard PCR was performed in a total volume of 25 µL containing 1 × HotStarTaq Master Mix (Qiagen AG, Hombrechtikon, Switzerland), 300 nM both primers (Gadlb-S, Gadlb-AS; Table 1), and 2.5 µL of the 1:100 diluted DNA.

Table 1. Overview on the different types of PCR performed for the development of the new real-time quantitative PCR (qPCR) assay for *Staphylococcus aureus* genotype B (GTB), including targets, primers, and probes

Target ¹	Amplicon size (bp)	Primer/probe name	Primers/probe sequence (5'–3')
Standard PCR			
RS-PCR	Various	G1 L1	GAA GTC GTA ACA AGG ² CAA GGC ATC CAC CGT ²
<i>adlb</i>	645	Gadlb-S Gadlb-AS	GTG ATG GAG GTA CTC TAA ATA CA ³ CCC AAA TAT TAT CTG CAT CT ³
Real-time qPCR			
lukEB	178	QlukEB-S QlukEB-AS	GGT GCT GAA GTA ATC AAA AGT ⁴ CAT ATC CAC TAC CCT TCA CAT C ⁴
<i>sea</i>	168	QlukE-P QseaM-S QseaM-AS	TG[A] GT[A] A[C]G [C]C[C] C[A]T ^{4,5} GCC GAT CAA TTT ATG GCT AGA C ⁴ CTC TGA ACC TTC CCA TCA AAA AC ⁴
<i>sed</i>	93	QseaM-P QsedM-S QsedM-AS QsedM-P	ACA GTA CCT TTG GAA ACG ^{4,6} TTA CCG TAC AAG AAT TAG ATG CAC AAG ⁴ GCT GTA TTT TTC CTC CGA GAG TAT CA ⁴ AAG GCG CTA TTT GC ^{4,6}
<i>adlb</i>	95	QadlbM-S QadlbM-AS QadlbM-P	TGC TAC ATT AGA TTT GCG TAC AGA TA ³ AGA CAT AGC GAC GAT CCT GAT TA ³ CAA CCT CAG CAT AGT AGT TA ^{3,6}
CDVN	130	QCDVN-S QCDVN-AS QCDVN-P	CAT CAA CTC TGT TTG CGG TCT TAC ⁷ CTT ATT CTC CAA CCA GCC TAA TTG T ⁷ CTC ATC TGC CTC AGA ATC CAA ACT TGC TC ^{7,8}
Melting curve PCR			
<i>nuc</i>	664	Nuc-S Nuc-AS	CTG GCA TAT GTA TGG CAA TTG TT ⁷ TAT TGA CCT GAA TCA GCG TTG TCT ⁷

¹RS-PCR = 16S-23S rRNA intergenic spacer region PCR; *adlb* = gene coding for the adhesion-like bovine protein; lukEB = SNP within the *lukE* gene (coding for leucotoxin E); *sea*, *sed* = genes of *Staphylococcus aureus* coding for the enterotoxins A and D; CDVN = N gene of the canine distemper virus; *nuc* = thermonuclease gene; S = sense primer; AS = antisense primer; P = probe.

²Reference: Jensen et al. (1993).

³Reference: this paper.

⁴Reference: Boss et al. (2011).

⁵Real-time quantitative PCR 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.

⁶Minor groove binder (MGB) qPCR probe labeled with FAM at the 5' end and with a confidential nonfluorescent quencher at the 3' end.

⁷Reference: Graber et al. (2007).

⁸Real-time quantitative PCR probe labeled with FAM at the 5' end and with 6-carboxytetramethylrhodamin (TAMRA) at the 3' end.

The Gadlb-S started at nucleotide 1,724, and Gadlb-AS ended at nucleotide 2,369; the resulting amplicon consists of 645 bp. The DNA amplification was carried out by a Biometra cycler system (Biometra GmbH, Göttingen, Germany). The cycling conditions of the standard PCR were an initial denaturation step (95°C, 10 min), 35 cycles comprising denaturation at 94°C, annealing at 54°C and elongation at 72°C, each for 1 min, followed by a final elongation step at 72°C for 10 min and cooling to 4°C. The PCR products were stored at -25°C until further processing and then analyzed on the miniaturized electrophoresis system Agilent DNA 7500 Chip (Agilent Technologies, Basel, Switzerland) to test for PCR amplification and the purity of the PCR product. Finally, PCR products were sent to Microsynth for purification and sequencing of the *adlb* gene; Gadlb-S was used as sequencing primer (Table 1).

Isolation of Bacterial DNA and Limit of Detection

Two different extraction procedures were used for the isolation of DNA from bacteria, depending on the purpose of the experiment.

Short Extraction Protocol (Boiling Preparation). To determine the inclusivity and exclusivity of the new qPCR assay, a short extraction protocol was applied. Strains from our strain collection, which were conserved in skim milk were grown overnight on BA at 37°C. One colony of each strain was picked from BA using a sterile plastic loop and inoculated in 100 µL of 10 mM Tris/HCl and 10 mM Na₂EDTA (pH = 8.5). Eppendorf tubes were incubated for 10 min at 95°C and then immediately placed on ice. A 1:100 dilution step of the DNA always preceded qPCR.

Simplified Extraction Protocol After Enrichment. The simplified extraction protocol according to Voelk et al. (2014) was used for the analysis of clinical milk samples.

Bacterial cultures were prepared by adding 500 µL of milk to 4.5 mL of Chapman medium [10 g/L of casein-peptone (Merck AG, Zug, Switzerland), 1 g/L of Lab-Lemco powder (Oxoid, Pratteln, Switzerland), 75 g/L of NaCl (Merck AG), 10 g/L of D-mannitol (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland)] and then incubated for 18 h at 37°C under constant shaking. After this bacterial enrichment step, DNA was extracted using the *mericon* DNA Bacteria Plus Kit (Qiagen AG), according to the instructions of the manufacturer (<https://www.qiagen.com/us/resources/resourcedetail?id=8a513dcc-dfc3-4101-bdb3-3f846b68ba87&lang=en>). In brief, 1 mL of bacterial culture was added to 650 µL of extraction mix, which contained 150 µL of *Lactobacillus casei* (10¹⁰ cfu/mL), 300 µL of Triton

X-100 (2%; Sigma-Aldrich Chemie GmbH), and 200 µL of Tris/HCl (100 mM, pH = 7.8). After centrifugation (18,000 × *g* for 5 min at 4°C), the supernatant was discarded using a vacuum pump. The bacterial pellet was resuspended in 400 µL of Fast Lysis Buffer (Qiagen AG) and, after transfer into the Pathogen Lysis Tubes (Qiagen AG), samples were horizontally vortexed for 10 min at maximal power. After centrifugation (13,000 × *g* for 5 min at 25°C), 100 µL of the supernatant was transferred into a new tube and used as template DNA for qPCR analysis.

The simplified extraction protocol was also used to determine the limit of detection of the new qPCR assay after enrichment of the milk samples. Starting from a liquid, log-phase culture of *Staph. aureus* GTB containing 2.35 × 10⁹ staphylococcal cell equivalents (SCE) per mL (V⁰), a 1:10 dilution series (V⁻¹ to V⁻¹⁰) was produced starting from V⁰ in Chapman medium. To simulate the enrichment of real milk samples collected in the field, 500 µL of each dilution (V⁻¹ to V⁻¹⁰) were incubated with 500 µL of cow raw milk (bacteriologically sterile) and 4 mL of Chapman medium (18 h, 37°C, under constant shaking). Finally, DNA was isolated following the procedure described above.

qPCR for *adlb*

Primers for qPCR are reported in Table 1. The sense primer (QadlbM-S) binds at position 2070 of the *adlb* gene and the antisense primer (QadlbM-AS) ends amplification at position 2165. The amplified region consists of 95 bp. The probe for *adlb* was minor groove binder-labeled with 6-carboxyfluorescein at the 5' end (reporter) and a confidential dark quencher at the 3' end (Boss et al., 2011) and was synthesized by Thermo Fischer Scientific. The qPCR reactions were run in a total volume of 25 µL, containing 1 × Roche LightCycler 480 Probes Master (Roche), 600 nM sense and antisense primer, 100 nM probe (QadlbM-P), and 3.5 µL of the DNA template. The DNA was isolated from the bacteria using 1 of the 2 isolation methods, as described previously. Amplification of the DNA was performed in a Rotor-Gene 6000 real-time thermal cycler (Corbett Life Science, Mortlake, Australia) under the following cycling conditions: after an initial denaturation step (95°C, 10 min), 45 cycles were run, including denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. According to Boss et al. (2011), qPCR reactions were always run in duplicate for the target gene *adlb*, considering as positive only those samples for which both reactions were positive. If only 1 reaction showed a positive result or the difference of the 2 cycle-to-threshold (Ct) values was more than 2 cycles, qPCR

Table 2. Bacterial strains analyzed for the determination of the inclusivity (97%) and exclusivity (98%) of the new real-time quantitative PCR assay for *Staphylococcus aureus* genotype B (percent in parentheses)

Bacterial strain ¹	n	Presence of <i>adlb</i> ²	
		Neg. ³	Pos. ³
GTB	99 (100)	3 (3)	96 (97)
Non-GTB			
GTC	24	24	0
GTOG	24	23	1
NSA	10	10	0
Streptococci	3	3	0
<i>Escherichia coli</i>	1	1	0
Total non-GTB	62 (100)	61 (98)	1 (2)

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

²Target gene: *adlb* = gene coding for the adhesion-like bovine protein.

³Neg. = number of negative real-time quantitative PCR results; Pos. = number of positive real-time quantitative PCR results.

was repeated. For each run, a qPCR standard curve was included according to Boss et al. (2011), ranging from 10 to 10⁷ copies/assay.

Assay Controls

According to Graber et al. (2007) and Boss et al. (2011), various controls were included to monitor the whole procedure. To evaluate the preparation of *Staph. aureus* GTB from milk (enrichment and DNA extraction controls), 1 sample of raw milk negative for *Staph. aureus* and 1 raw milk sample spiked with approximately 10⁵ cfu/mL of *Staph. aureus* GTB were always co-processed with the test samples (Graber et al., 2007; Boss et al., 2011). If the negative raw milk sample showed amplification or if the spiked sample was not within the adequate range (± 1 cycle of the expected Ct-value), all test samples of the affected series were prepared again (Boss et al., 2011). This was never necessary during our experiments.

For each qPCR run, 1 no-template control and 1 *Staph. aureus* GTB-positive control were included. If the results were positive for no-template control or negative for positive control, the run was repeated. To check for qPCR inhibitors present in the DNA, samples were always analyzed by an additional qPCR containing amplicons of the N gene of the canine distemper virus (10³ copies/assay) as an internal control. Negative qPCR results for the *adlb* target sequence required a positive qPCR result for the N gene of the canine distemper virus to exclude inhibitors (Graber et al., 2007; Boss et al., 2011).

Bacterial Strains

To evaluate the inclusivity and exclusivity of the new qPCR assay, a selection of udder pathogens with known

identity from our strain collection were tested for the presence of the target gene *adlb* (Table 2). All bacterial strains used for this purpose were originally isolated from aseptically collected bovine milk samples and then stored in skim milk at -25°C . For isolation of the DNA, bacteria were recultured on BA and incubated overnight at 37°C .

A total of 147 *Staph. aureus* strains were included in the study. All strains were obtained from previous studies (Graber et al., 2007; Fournier et al., 2008; Cosandey et al., 2016) and were randomly selected. They were isolated from bovine milk samples with spontaneous *Staph. aureus* IMI and were epidemiologically unrelated, meaning that they originated from different farms. Out of them, 99 strains were GTB, 24 were GTC, and 24 were GTOG; all resulted positive for the *Staph. aureus*-specific *nuc* gene (Brakstad et al., 1992; Graber et al., 2007).

A selection of 10 non-*Staph. aureus* staphylococci (NSA) were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), including *Staphylococcus saprophyticus* ssp. *saprophyticus* (DSM 20229), *Staphylococcus epidermidis* (DSM 20044), *Staphylococcus haemolyticus* (DSM 20263), *Staphylococcus xylosus* (DSM 20266), *Staphylococcus warneri* (DSM 20316), *Staphylococcus intermedius* (DSM 20373), *Staphylococcus chromogenes* (DSM 20454), *Staphylococcus hyicus* (DSM 20459), *Staphylococcus caprae* (DSM 20608), and *Staphylococcus simulans* (DSM 20322). Furthermore, 3 streptococcal strains (*Streptococcus uberis*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae*) and 1 *Escherichia coli* strain were included. These strains were isolated from quarters with spontaneous IMI during a previous study by Wyder et al. (2011). The 3 streptococcal strains were identified by PCR, as de-

scribed by Raemy et al. (2013), as well as the *E. coli* strain, according to Riffon et al. (2001).

Spiking Experiments

A possible negative influence of foreign (NSA) DNA on the amplification of the *adlb* gene was investigated by analyzing *Staph. aureus* GTB DNA samples (10^4 to 10^7 SCE/assay) after addition of DNA extracts from 3 different NSA (*Staph. chromogenes*, *Staphylococcus sciuri*, and *Staph. xylosus*, at concentrations of 10^2 to 10^7 SCE/assay) into the master mix used for qPCR. The DNA of all strains used for this experiment were isolated using the boiling prep protocol, followed by DNA purification using the High Pure PCR Template Preparation Kit (Roche). All combinations of *Staph. aureus* GTB and each NSA were separately analyzed by qPCR.

Diagnostic Sensitivity and Specificity (Clinical Milk Samples)

Milk samples analyzed to determine the diagnostic sensitivity and specificity of the new qPCR assay originated from 20 GTB-positive dairy herds, which participated in a longitudinal field study aiming to eradicate *Staph. aureus* GTB from the milking herd (C. Sartori, unpublished data). The within-herd prevalence for *Staph. aureus* GTB in those herds was between 5 and 57%, with a median of 20% (GTB-positive cows per herd). After cow preparation for milking by the farming personnel (including forestripping and teat cleaning), samples were collected either under clean conditions (composite foremilk samples of each cow) or under aseptic conditions (single-quarter foremilk samples, which were then pooled in the laboratory to form a composite milk sample of each cow).

During the first sampling of all herds (median herd size = 25 cows, with a minimum of 11 and a maximum of 108 cows/herd) participating in the sanitation study, a total of about 500 single-cow milk samples was collected. Of those, 241 milk samples were randomly selected (including both GTB-positive and -negative samples in approximately equal amounts) and analyzed using 2 different qPCR assays: (1) the new qPCR assay based on the detection of the unique target gene *adlb* was compared with (2) the established qPCR assay by Boss et al. (2011), which was used as a reference and relies on the detection of the 3 targets *lukEB*, *sea*, and *sed*. A positive result for *Staph. aureus* GTB is given when the targets *lukEB* and *sea* or *sed* (or both) are positive (Boss et al., 2011), or when the unique target gene *adlb* is positive (new qPCR assay). According to Syring et al. (2012), a reaction was considered positive

if the amplification of the targets *lukEB*, *sea*, and *sed* resulted in a value of 1.21×10^4 copies/assay. For the new qPCR assay, a reaction was considered positive if the amplification of the unique target gene *adlb* resulted in a value of 9.25×10^4 copies/assay, corresponding to a Ct value of 21.8. The cut-off Ct value for a positive qPCR result was calculated (according to Bustin et al., 2009; Caraguel et al., 2011) by adding 3 times the standard deviation to the mean value of all positive and always replicable results (dilution V^{-4}) obtained during the repeatability experiments.

In the case of a discrepancy in the final result (samples being positive with only 1 of the 2 qPCR assays), samples were further analyzed by 16S-23S rRNA intergenic spacer region PCR (RS-PCR; Fournier et al., 2008). For this purpose, the originally collected milk samples were thawed and 20 μ L of each sample were plated on BA and incubated overnight at 37°C. For samples showing no growth after incubation, an enrichment step was performed (1:10 dilution of the milk sample in Chapman medium, in a total volume of 5 mL) and the enriched samples were then plated on BA and processed identically to the unenriched samples.

From each BA plate, 5 colonies were randomly selected and DNA was isolated using the boiling preparation method. For the definitive identification of *Staph. aureus*, DNA was diluted 1:100 and melting curve PCR was performed for each sample for the target gene *nuc*.

Each *nuc*-positive sample was finally analyzed by RS-PCR (Fournier et al., 2008), which is the gold-standard method for genotyping. This includes, in brief, the amplification of the 16S-23S rRNA intergenic spacer region by PCR (Fournier et al., 2008), followed by the analysis of the PCR products using the miniaturized electrophoresis system Agilent DNA 7500 Chip (Agilent Technologies). This system separates amplicons of DNA according to their size, which results in a plot of correspondent peaks (electropherogram) that can be monitored on a personal computer, evaluated, and translated in a pseudo-gel by a particular software (Agilent Technologies, according to Fournier et al., 2008). The interpretation of the resulting electropherograms was performed using the in-house developed Mahal program (Syring et al., 2012).

Repeatability

Interassay Variability. To determine the interassay variability of the new qPCR assay, serial 1:10 dilutions of *Staph. aureus* GTB were completely analyzed 20 times. Five complete analysis rounds of the same dilution series were performed per working day, on each of 4 consecutive days. All samples were independently processed following all steps of the analytical procedure

(bacterial enrichment, DNA extraction, and qPCR analysis). Dilution series (V^{-1} to V^{-10}) were produced starting from a liquid, log-phase culture (V^0) of *Staph. aureus* GTB. For bacterial enrichment, 500 μ L of each dilution and 500 μ L of cow raw milk (bacteriologically sterile) were added to 4 mL of Chapman medium and incubated for 18 h at 37°C under constant shaking. Thereafter, DNA was isolated using the simplified extraction protocol and, finally, all samples of each dilution series were analyzed by qPCR for the target gene *adlb* on 20 different runs.

Intra-Assay Variability. To determine the intra-assay variability of the new qPCR assay, a 1:10 dilution series (V^{-1} to V^{-6}) of *Staph. aureus* GTB was prepared and analyzed as described for the interassay experiment. After bacterial enrichment, the DNA was independently isolated 5 times from each of the 6 dilutions using the same extraction mix and the simplified protocol. Finally, each sample was analyzed for *adlb* on the same qPCR run, using a new qPCR master mix for each of the 5 subseries (V^{-1} to V^{-6}).

Statistical Analysis

Data were expressed as absolute or relative frequencies (%). Inclusivity, exclusivity, as well as diagnostic sensitivity and specificity were calculated in the standard manner (OIE, 2016b). A 2-factor ANOVA was performed to compute the overall standard deviation of the intra-assay variability expressed in Ct values. The 2 independent factors were sample dilution and qPCR master mix. As a post hoc test, the Scheffé test was applied. For all the statistical analyses, the Systat 13.1 software package (Systat Software, San Jose, CA) was used. Values of $P < 0.05$ were considered to be significant.

RESULTS

Characterization of the Target Gene

The target gene of the GTB strains M5512B and M6020B were both 3,798 bp long, resulting in a protein of 1,265 AA. It includes an N-terminal gram-positive signal peptide at AA 17 to 54 and a C-terminal gram-positive Leu-Pro-any-Thr-Gly cell wall anchor at AA 1,219 to 1,257. Furthermore, the protein harbors 3 G5 domains (AA 842–921, AA 960–1,042, AA 1,088–1,170); in between, 2 E domains are located (AA 914–961, AA 1,042–1,089). The G5 domains are involved in bacterial adhesion so that both the protein and gene were named adhesion-like bovine protein (*adlb*).

Figure 1. Standard curve obtained for real-time quantitative PCR (qPCR) using the *adlb* gene as the target. Serial dilutions of a purified *adlb* gene amplicon were made starting at 10^7 staphylococcal cell equivalents (SCE)/assay and ending at 10 SCE/assay. The dilutions were amplified by the standard *adlb* gene qPCR protocol, and the corresponding cycles to reach a defined threshold (Ct) were determined. The SCE/assay values ($\log_{10}$ scale) were then plotted against the obtained Ct values. The observed coefficient of correlation was $R = -0.999$. The equation describing the standard curve was $Ct = -3.6734 \times \log_{10} (\text{SCE/assay}) + 40.08$.

qPCR Assay Performance

Using serial dilutions of a purified *adlb* gene amplicon, the assay was shown to give linear results between 10 and 10^7 SCE/assay. As a qPCR standard, therefore, we chose concentrations between 10 and 10^7 SCE per run. For this range, the coefficient of correlation was -0.999 (Figure 1). The amplification efficiency was 0.91, as measured on the gradient.

Analytical Specificity

Inclusivity. Out of the 99 *Staph. aureus* GTB strains analyzed to determine the inclusivity of the new qPCR assay, 96 strains resulted positive for *adlb*, whereas 3 strains were negative, resulting in an inclusivity of 97% (95% CI = ± 2) for the new target gene (Table 2).

Exclusivity. Out of the 62 strains other than *Staph. aureus* GTB analyzed to determine the exclusivity of the new qPCR assay, all the *Staph. aureus* GTC strains, as well as the streptococci, the *E. coli*, and all the NSA strains were found to be negative for *adlb*. Only 1 GTOG strain was positive for *adlb*, resulting in

an exclusivity of 98% (95% CI = ± 2) for the new target gene *adlb* (Table 2).

Spiking Experiments

No systematic shift of the *adlb* amplification signals (Ct values) was observed for any of the 6 amounts of DNA of each NSA compared with those of the non-spiked samples, which represented the reference value. The range of variation between the Ct values of spiked and nonspiked samples was 0.23 cycles for *Staph. chromogenes*, 0.45 cycles for *Staph. sciuri*, and 0.58 cycles for *Staph. xylosus*.

Analytical Sensitivity

The limit of detection of the novel assay was evaluated by analyzing serial 1:10 dilutions of a *Staph. aureus* GTB culture after enrichment, according to the standard procedure as used for inferring the interassay variability (see below). Out of 5 repetitive dilution experiments, 3 gave a positive qPCR result for V^{-7} , which

corresponds to a detection limit of 2.35×10^2 SCE/mL before enrichment.

Repeatability

Interassay Variability. Replicable, positive results were obtained for all 20 analysis rounds until V^{-4} , corresponding to 2.35×10^5 SCE/mL before enrichment. Below this concentration, repetition was no longer consistent but was observed from time to time, as was the case when inferring the limit of detection of the assay.

Intra-Assay Variability. This experiment showed replicable results for the 4 dilutions V^{-1} to V^{-4} , which contained, respectively, 3.25×10^8 to 3.25×10^5 SCE/mL before enrichment. The ANOVA, which considered the 2 independent factors dilution and qPCR master mix, resulted in an overall standard deviation of 0.59 cycles for the measured Ct values. The factor qPCR master mix had no influence on the Ct values, meaning that no shifts could be observed in the amplification curves when analyzing the same dilution using 5 different qPCR master mixes on the same qPCR run. As expected, the factor dilution had a significant effect ($P < 0.001$) on the Ct values, resulting in higher Ct values for lower dilutions (Figure 2). Mean Ct values were shifted 0.12 cycles for V^{-2} ($P = 0.976$), 0.88 for V^{-3} ($P = 0.022$), and 3.78 cycles for V^{-4} ($P < 0.001$) compared with baseline Ct values for V^{-1} .

Figure 2. Positive real-time quantitative PCR (qPCR) results after milk sample enrichment and cut-off for a positive qPCR result. Samples contained 3.25×10^8 to 3.25×10^5 staphylococcal cell equivalents (SCE)/mL before enrichment and were analyzed in duplicate. The resulting cycles to reach the threshold (Ct values) showed a relatively narrow range of variation ($17.13 > Ct > 12.33$), independent of the bacterial concentration present in the samples before enrichment. The vertical line (at Ct = 21.8) represents the cut-off value for a positive qPCR result. Norm. Fluoro. = normalized fluorescence.

Diagnostic Sensitivity and Specificity (Clinical Milk Samples)

A total of 241 milk samples were randomly selected and analyzed to determine the diagnostic sensitivity and specificity of the new *adlb* test. Both analytical methods, the reference qPCR assay including the targets *lukEB*, *sea*, and *sed* as well as the new *adlb* assay, delivered identical results for 225 samples (109 GTB-positive and 116 GTB-negative). A total of 16 samples showed discordant results using the 2 methods mentioned above. For 15 GTB-negative samples with the reference method, positive results were obtained by the *adlb* assay. In contrast, 1 sample was GTB-negative by the new *adlb* assay and GTB-positive by the reference method. All samples showing discordant results were further analyzed by RS-PCR (gold-standard genotyping method), whereby all of them generated a GTB-positive result. The total number of correct GTB-positive results was therefore 125 (109 + 16). Of those, 124 samples were identified as GTB-positive by the *adlb* method, resulting in a diagnostic sensitivity of 99% (95% CI = ± 2). As all the GTB-negative samples were correctly identified, the diagnostic specificity for the new target gene *adlb* was 100% (95% CI = ± 2).

DISCUSSION

The aim of the present study was the development, technical evaluation, and validation of a new diagnostic qPCR assay for the detection of *Staph. aureus* GTB in dairy cow milk samples. This mastitis pathogen is contagious (Graber et al., 2009; van den Borne et al., 2017) and particularly prevalent in Switzerland (Fournier et al., 2008; Graber et al., 2009) and other central European countries, including Switzerland's neighboring countries (Austria, France, Germany, and Italy) and Belgium (Cosandey et al., 2016). The new test is based on the detection of the unique target gene *adlb*, which results in a considerable simplification compared with the GTB diagnostics currently in use (Boss et al., 2011; Syring et al., 2012), as the latter requires the parallel detection of 3 GTB-typical targets (Boss et al., 2011): *lukEB*, coding for a SNP within the *lukE* gene, and the genes *sea* and *sed*, coding for staphylococcal enterotoxins A and D, respectively. The new procedure relies on a rapid isolation of the bacterial DNA from enriched milk samples using a commercial kit and the qPCR for the new target gene *adlb*, which is highly inclusive and exclusive for *Staph. aureus* GTB. Additionally, the new qPCR assay shows a high analytical sensitivity and is characterized by an excellent diagnostic sensitivity and specificity, enabling a very reliable detection of GTB-positive cows and a clear exclusion when the test is negative. Because it analyzes a single target gene, the new analytical procedure is particularly suitable for routine application requiring high throughput.

Inclusivity and Exclusivity

The 3 *adlb*-negative strains were unrelated and had different origins. Two strains were obtained from clinical milk samples of subclinical IMI collected in Switzerland and 1 strain was isolated from a sample collected in Austria during the European study by Cosandey et al. (2016). One possible reason for the negativity of these 3 strains for the new target is the loss of the gene *adlb*. This could be the consequence of the strain conservation process, as described by van Griethuysen et al. (2005) for the loss of the *mecA* gene in *Staph. aureus* after 2 yr of storage at -80°C . The only non-GTB strain being positive for *adlb* was *Staph. aureus* of genotype N. Based on our own bioinformatics analyses, this genotype was found to be genetically very close to *Staph. aureus* GTB (H. U. Graber, unpublished data). Compared with the qPCR assay previously developed by Boss et al. (2011), which was characterized by 100% inclusivity and exclusivity, we obtained slightly lower values. However, this may be interpreted as a negligible difference, considering the higher total number

of strains selected for analysis in our study ($n = 161$) versus the previous study ($n = 116$; Boss et al., 2011).

Spiking Experiments

Spiking experiments were carried out to investigate the influence of foreign (NSA) DNA on the qPCR signals (Ct values) of the new qPCR assay. To quantify a possible disturbance effect of foreign bacterial DNA during the detection of the target organism *Staph. aureus* GTB, 3 NSA strains typically found in clinical milk samples (*Staph. chromogenes*, *Staph. sciuri*, and *Staph. xylosus*; Taponen and Pyorala, 2009; Supré et al., 2011; Vanderhaeghen et al., 2014) were selected and individually spiked in different concentrations (10^7 to 10^2 SCE/assay) in the qPCR master mix used to analyze GTB-positive samples spiked with different concentrations of the target organism (10^7 to 10^4 SCE/assay). The results of our experiment confirmed that the new qPCR assay for *Staph. aureus* GTB is reliable also in the case of samples containing high concentrations of foreign bacterial DNA. This reflects possible field conditions of *Staph. aureus* GTB-positive cows additionally colonized with other typical IMI-related bacteria (e.g., other staphylococci). With a maximal range of variation of 0.58 cycles between the qPCR signals of samples containing only the DNA of *Staph. aureus* GTB and of those additionally spiked with NSA DNA, the effect of foreign DNA was minimal over the whole range of tested *Staph. aureus* GTB concentrations. The presence of foreign DNA in the samples never led to false-negative results for the target organism *Staph. aureus* GTB. This was true independent of the strain used for spiking (*Staph. chromogenes*, *Staph. sciuri*, and *Staph. xylosus*) and of the spiked concentration, meaning that the new qPCR assay is reliable even if the milk samples subjected to GTB analysis are highly contaminated with other staphylococcal species. According to previous studies (Boss et al., 2011; Syring et al., 2012; Voelk et al., 2014), the observed Ct differences were considerably lower than the maximal range of variation of 2 cycles, which is considered acceptable when analyzing the same sample by qPCR in duplicates. According to these results, we deduced that the presence of NSA in the milk, even in high concentrations, does not affect the stability of the new qPCR assay. This advantageous property of the new diagnostic approach makes it suitable for use as a routine test, even if cows are simultaneously infected with different mastitis pathogens.

Analytical Sensitivity

The limit of detection for the present assay was 2.35×10^2 SCE/mL, which indicates the minimal amount of

Staph. aureus GTB to be contained in a liquid before enrichment, enabling to generate a positive qPCR result after enrichment. The observed limit of detection of the novel assay corresponds to about 34 cfu/mL, considering an average conversion factor of 7, as described by Graber et al. (2007). The analytical sensitivity of the present test is in line with that found by Boss et al. (2011) by isolating *Staph. aureus* GTB directly from milk. Indeed, their limits of detection were 40 cfu/mL for lukEB, 100 cfu/mL for *sea*, and 10 cfu/mL for *sed*.

Repeatability

The repeatability of the present assay seems to considerably depend on the amount of *Staph. aureus* GTB in the milk sample to be analyzed. In fact, both inter- and intra-assay experiments showed highly replicable results until V^{-4} , which corresponded to 2.35×10^5 and 3.25×10^5 SCE/mL of culture before enrichment, respectively.

For concentrations $\geq 3.25 \times 10^7$ SCE/mL, the resulting Ct values were identical, meaning that under these conditions bacterial growth ended up in a plateau (Figure 2). For a concentration of 3.25×10^6 SCE/mL, the mean Ct value was significantly higher than for the higher concentrations, but they were still very similar. A considerable Ct shift, however, was observed for 3.25×10^5 SCE/mL, which also turned out to be the minimal replicable concentration.

The reasons for the high minimal bacterial concentration (2.35×10^5 SCE/mL) required in the sample to obtain a replicable result and for the inconsistent results at lower concentrations are not completely clear. Apparently, various and probably interacting factors may influence the ability of bacteria to grow during enrichment. Based on the results of our repeatability experiments, the growth of *Staph. aureus* GTB in Chapman medium seems to strongly depend on having reached the critical concentration of about 10^5 SCE/mL. The reason for this phenomenon remains largely unclear. What is striking is the fact that the critical concentration is the same as the one required for enterotoxin production (Paulin et al., 2011), suggesting a regulation by the quorum-sensing mechanism (Paulin et al., 2011). For a better understanding of the observed phenomenon, however, further study is required addressing the influence of various factors and mechanisms on growth of *Staph. aureus* GTB in culture.

Diagnostic Sensitivity and Specificity (Clinical Milk Samples)

To determine the diagnostic sensitivity and specificity of the new qPCR assay, 241 representative milk sam-

ples were randomly selected from an ongoing sanitation study for *Staph. aureus* GTB and analyzed using both the reference qPCR assay by Boss et al. (2011) and the new *adlb* assay. Identical results were obtained for 93% of the samples, whereas for the remaining 7% discordant results were observed. These samples were further analyzed using the gold-standard method by Fournier et al. (2008), identifying all 16 strains as *Staph. aureus* GTB. Of those, 15 turned out to be GTB-positive by the *adlb* assay. This means that the new qPCR assay shows a higher diagnostic sensitivity than the one by Boss et al. (2011), demonstrating a relevant improvement of the GTB diagnostics. In fact, these 15 milk samples were exclusively positive for the *adlb* gene but negative for both enterotoxin genes *sea* and *sed*. These samples would be falsely classified as GTB-negative using the current qPCR assay by Boss et al. (2011), with the consequence that the contagious problem would persist in the herd. A further consequence is that the sanitation of whole regions would be slowed down due to new infections remaining undetected by this analytical method. With a diagnostic sensitivity of 99% and a specificity of 100%, the novel assay therefore enables detection of GTB-positive and GTB-negative cows highly reliably, reducing the risk for false-negative and false-positive results in clinical milk samples to a minimum.

CONCLUSIONS

The new qPCR assay for the detection of *Staph. aureus* GTB in cow milk is based on the analysis of the unique target gene *adlb*, which shows high inclusivity, exclusivity, and sensitivity at the analytical level, as well as excellent diagnostic properties. All steps of the analytical procedure (sample collection and enrichment, DNA extraction, and qPCR analysis) are straightforward and can be performed under standardized conditions. The new test can be carried out routinely, enabling laboratories to manage high numbers of milk samples. Because of these properties, the new diagnostic tool is particularly suitable for the decision-making process during sanitation programs for the contagious mastitis pathogen *Staph. aureus* GTB. The analysis of a unique target gene accelerates the screen of whole herds, allowing farmers and veterinarians to immediately apply preventive or control measures (segregation, therapy, culling) after the detection of infected cows, keeping the risk of new infections under control.

ACKNOWLEDGMENTS

The study was supported by a research grant of the Federal Office for Agriculture (FOAG, Berne, Swit-

zerland), and by the Swiss National Science Foundation (SNSF, Berne, Switzerland), project number 310030_143650.

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