Differentiation of *Staphylococcus aureus* and *Staphylococcus epidermidis* by PCR for the fibrinogen binding protein gene

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ABSTRACT

Mastitis is one of the most common and burdensome diseases afflicting dairy animals. Among other causes of mastitis, staphylococci are frequently associated with clinical and subclinical mastitis. Although *Staphylococcus aureus* is the predominant species involved, *Staphylococcus epidermidis* and other coagulase-negative staphylococci are increasingly being isolated from cases of bovine mastitis. Although *Staph. aureus* and *Staph. epidermidis* can be easily differentiated based on their biochemical properties, such phenotypic identification is time consuming and laborious. This study aimed to rapidly identify *Staph. aureus* and *Staph. epidermidis*. Accordingly, a multiplex PCR was developed and we found that a single gene encoding the adhesin fibrinogen binding protein could be used to identify and differentiate the two species. Consequently, a multiplex reaction combining a triplex PCR for *Staph. aureus* and a duplex PCR for *Staph. epidermidis* was standardized, first using bacterial cultures and then with pasteurized milk spiked with live organisms or DNA extracted from the organisms. The test could specifically detect *Staph. aureus* and *Staph. epidermidis* even in the presence of a dozen other organisms. The limit of detection for detecting *Staph. aureus* and *Staph. epidermidis* separately was 10 to 100 cfu/mL for simplex PCR and 10⁴ cfu/mL for multiplex PCR. Conversely, the limit was 10⁶ cfu/mL by multiplex PCR for simultaneous detection of both the organisms when spiked into culture medium or pasteurized milk. Overnight enrichment enhanced the assay sensitivity 100-fold. The assay had a high

diagnostic sensitivity and specificity. The application of the test was verified on 602 field isolates of staphylococci that had been characterized earlier by phenotypic methods. Importantly, 25 coagulase-negative isolates were identified as *Staph. aureus* by the multiplex PCR. The test could be adapted for use in clinical diagnostic laboratories.

Key words: bovine mastitis, staphylococci, detection and differentiation, multiplex PCR

INTRODUCTION

Bovine mastitis is one of the most important diseases affecting dairy industry. The disease costs around Rs 72 billion (approximately \$1.4 billion) per year (Bansal and Gupta, 2009) to India, where the livestock sector contributes 5 to 6% to the gross domestic product. Speedy diagnosis of mastitis, particularly that of subclinical cases, and rapid identification of the causative agent(s) are crucial for designing appropriate therapy and management to control mastitis.

Bacteria account for most IMI. During mastitis, multiple pathogens, variable shedding, inhibitors from both the host and the microbes, antibiotic residues, presence of inflammatory cells, and the persistence of inflammation even after the clearance of bacteria produce a dynamic state of udder infection and inflammation (Pyörälä and Mattila, 1987; Sears et al., 1990; Dinsmore et al., 1992; Suojala et al., 2008; Taponen et al., 2009; Koskinen et al., 2010). These and other factors, such as unfavorable conditions during transport of samples and during enrichment (Dinsmore et al., 1992; Koskinen et al., 2010), incubation or freezing before culturing, inoculum volume, and enrichment conditions (Schukken et al., 1989; Dinsmore et al., 1992; Gillespie and Oliver, 2005; Taponen et al., 2009), can influence

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isolation and identification of bacteria. Further, phenotyping methods are time consuming, have low sensitivity, lack interlaboratory standardization, and are not highly reliable (Pyörälä and Mattila, 1987; Sears et al., 1990; Phuektes et al., 2001; Pitkälä et al., 2005; Suojala et al., 2008), necessitating the development of better tests.

Among the known and potential pathogens that account for most IMI, only a few species of staphylococci predominate (Wilson et al., 1997; Tenhagen et al., 2006). Staphylococci can establish infection rapidly, and frequently persist and remain undetected for long periods particularly in subclinical mastitis. Phenotypic methods remain the gold standard for the identification of staphylococci, and several culture-based systems have been commercialized. However, 30 to 50% of mastitic milk samples may not show any bacterial growth under conventional culture conditions (Dinsmore et al., 1992; Makovec and Ruegg, 2003; Bradley et al., 2007; Olde Riekerink et al., 2008; Taponen and Pyörälä, 2009). In addition, phenotypic methods have poor discriminatory power, as they frequently misidentify species due to overlapping phenotypes (Renneberg et al., 1995; Rhoden and Miller, 1995; Pitkälä et al., 2005; Zadoks and Watts, 2009).

Polymerase chain reaction analysis has several advantages over phenotypic methods, including (1) direct detection and identification without culturing, (2) rapid turnaround time, (3) high throughput capability, (4) high accuracy, reproducibility, and sensitivity or specificity, and (5) objective user-independent interpretation. Single and multiplex PCR using both end-point and real-time methodologies, either alone or in combination with other techniques, have been applied for the identification of staphylococci to genus and species level, as well as to predict the ability of the organisms to express toxins or virulence determinants or resistance to antibiotics (Mehrotra et al., 2000; Martineau et al., 2001; Strommenger et al., 2003; Tristan et al., 2003; Morot-Bizot et al., 2004; Giammarinaro et al., 2005; Lee et al., 2008; Sasaki et al., 2010; Hirotaki et al., 2011; Shome et al., 2011; Chiang et al., 2012a). Various genes that have been probed either independently or in combination include *16S rRNA*, *23S rRNA*, *clfA*, *coa*, *cpn60*, *femA/B*, *gap*, *gla*, *groESL*, *hsp60*, *ITS*, *nuc*, *orfX*, *rdr*, *rpoB*, *sodA*, *spa*, and *tuf*, as well as the Sa442 and Se705 fragments (Martineau et al., 1996; 1998; Van-nuffel et al., 1999; Couto et al., 2001; Mason et al., 2001; Ghebremedhin et al., 2008; Hirotaki et al., 2011; Shome et al., 2011; Chiang et al., 2012a). Several molecular diagnostic kits have also been commercialized.

Staphylococcus aureus is the most important species among the mastitis-associated staphylococci (Jain, 1979; Wilson et al., 1997). This is followed by a group

of CNS (Pyörälä and Taponen, 2009), most importantly *Staphylococcus epidermidis*, in addition to *Staphylococcus chromogenes*, *Staphylococcus simulans*, and *Staphylococcus hemolyticus*. In a recent pilot study, we found that the frequency of isolation of *Staph. aureus* was more in individual households and that CNS were more frequent in an organized dairy sector in a small region in South India (Hegde et al., 2012). We reasoned that the pathobiology of mastitis caused by *Staph. aureus* or CNS could be different, and that rapid identification and differentiation of *Staph. aureus* and *Staph. epidermidis* could assist in designing of differential intervention strategies. During bioinformatics analyses to develop PCR for simultaneous detection of virulence determinants and differentiation of mastitis-associated *Staph. aureus* and *Staph. epidermidis*, we found that the gene encoding the fibrinogen binding protein, *fib*, can differentiate the 2 species. The development appeared in a single reaction of a multiplex PCR assay for concurrent detection and differentiation of *Staph. aureus* and *Staph. epidermidis*. The results indicate that these assays could be adapted for the detection of these organisms in milk.

MATERIALS AND METHODS

Bioinformatics Analyses

Complete or partial genomes of *Staph. aureus*, *Staph. epidermidis*, *Staph. hemolyticus*, and *Staphylococcus saprophyticus* were aligned using ClustalW program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Unique primers were designed for *fib* and thermonuclease (*nuc*) genes using the PrimerQuest program (Integrated DNA Technologies, Coralville, IA).

Bacterial Strains and Biochemical Methods

All media and reagents for microbiological assays were obtained from HiMedia Laboratories (Mumbai, India). Reference strains of bacteria were obtained from Microbial Type Culture Collection (Chandigarh, India). Field isolates of staphylococci were obtained from clinical and subclinical bovine mastitis samples. Species identification was performed by colony and microscopic morphology, sugar fermentation, and coagulase, phosphatase, and thermonuclease activities (Sundareshan et al., 2012). The identified *Staph. aureus* and *Staph. epidermidis* strains were maintained at -80°C in brain-heart infusion broth supplemented with 20% glycerol (vol/vol).

Extraction of DNA, Primers, and PCR Conditions

For routine PCR, bacterial genomic DNA was extracted from overnight culture of a single colony by

using RealGenomics DNA isolation kit (Real Biotech Corporation, Chennai, India). For limit of detection experiments, 1 mL of overnight culture was used without dilution or serially diluted 10-fold to 1 mL in PBS. Genomic DNA was prepared by heating the culture or diluted sample in boiling water for 10 min, harvesting the supernatant, and extracting the DNA (Rawool et al., 2007). For experiments without or with enrichment, known colony-forming units of bacteria were inoculated into milk or milk plus brain-heart infusion broth (1:1), and DNA was extracted following overnight incubation at 37°C.

All PCR-related materials, including primers, were obtained from Sigma-Aldrich (Bengaluru, India). Primers were selected based on published information or designed through bioinformatics analyses (Table 1). The PCR was carried out as follows: initial 5-min denaturation step at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C, with a 10-min final extension at 72°C. The specificity of each pair of primers was verified by testing against the DNA of standard strains. Multiplex PCR was standardized by varying the combination of primer pairs, annealing temperature, and concentrations of primers, deoxynucleotide triphosphates, and enzyme.

Analytical and Diagnostic Parameters

Limit of detection (**LOD**) was determined by serially diluting ($\log_{10}$ -fold) cultures with known counts (cfu) of *Staph. aureus* or *Staph. epidermidis* in PBS or pasteurized milk and subjecting to multiplex PCR. The LOD was determined to be the final $\log_{10}$ dilution of the template in which the product was clearly visible after agarose gel electrophoresis.

True and false positives and negatives for the multiplex PCR (test) were deduced by comparison with culture and biochemical identification (standard). Samples corroborating the standard in the positive and negative pool were identified as true, those that did not conform to the results of the standard were identified as false. From the resulting data, the following parameters were calculated as indicated and converted to percentages:

$$\text{Analytical sensitivity} = \text{LOD},$$

$$\text{Analytical specificity (accuracy)} = \frac{\text{[no. test positive]}}{\text{[no. standard positive]}},$$

$$\text{Diagnostic (clinical) sensitivity} = \frac{\text{[no. true positive]}}{\text{[no. true positive + no. false negative]}},$$

$$\text{Diagnostic (clinical) specificity} = \frac{\text{[no. true negative]}}{\text{[no. true negative + no. false positive]}},$$

$$\text{Positive predictive value} = \frac{\text{[no. true positive]}}{\text{[no. true positive + no. false positive]}},$$

$$\text{Negative predictive value} = \frac{\text{[no. true negative]}}{\text{[no. true negative + no. false negative]}}.$$

RESULTS

Standardization of Simplex PCR

While comparing 15 *Staph. aureus* and 2 *Staph. epidermidis* genomes to select primers for virulence genes,

Table 1. Oligonucleotide primers used in the present study

Primer ¹	Sequence (5'-3') ²	Length (bp)	Reference
<i>23S rRNA</i>	ACGGAGTTACAAAGGACGAC (For) AGCTCAGCCTTAACGAGTAC (Rev)	1,250	Straub et al. (1999)
<i>FnBp</i>	AGCACAAGGACCAGTCGAGGAAAT (For) TCTTCTTTGGCAGGTGGTACTGGT (Rev)	750	This study
<i>Sa-fib</i>	AATTGCGTCAACAGCAGATGCCGAG (For) GGACGTGCACCATATTGCAATGTACC (Rev)	210	This study
<i>Nuc</i>	GTGCTGGCATATGTATGGCAATTGT (For) TCTTTGACCTTTGTCAAACCTCGA (Rev)	441	This study
<i>Se-fib</i>	AGTACAGAACCGTTATGCCTGGCT (For) TGATGAGTCAATTGCTGCTCCCGT (Rev)	720	This study
<i>Se-124</i>	ATCAAAAAGTTGGCGAACCTTTTCA (For) CAAAAGAGCGTGGAGAAAAGTATC (Rev)	124	Martineau et al. (1996)
<i>Eubac</i>	GGAGGAAGGTGGGGATGACG (For) ATGCTGTGACGGGCGGTGTG (Rev)	240	Martineau et al. (1996)

¹The eubacterial 16S rRNA primers were used separately to confirm the presence of genomic DNA.

²For = forward; Rev = reverse.

Figure 1. Products amplified by simplex or multiplex PCR on *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates were subjected to agarose gel electrophoresis. Lane 1 = *23S rRNA*; lane 2 = *nuc*; lane 3 = *Sa-fib*; lane 4 = *Se-fib*; lane 5 = *Se-124*; lane M = DNA marker; lane 6 = multiplex PCR of *Staph. aureus* with *23S rRNA*, *nuc*, and *Sa-fib* specific primers; lane 7 = duplex PCR of *Staph. epidermidis* with *Se-fib* and *Se-124*; and lane 8 = multiplex PCR with all primers using *Staph. aureus* and *Staph. epidermidis* templates.

we identified short stretches within the sequences of *fib* to be able to differentiate the two species. Primers for both the species as well as for differing lengths of fibrinogen binding protein (*FnBp*) and *nuc* fragments were designed to suit multiplexing (Table 1).

Initial amplification of individual fragments was standardized using reference strains. Fragments of *23S rRNA* (1,250 bp), *nuc* (441 bp), and *Sa-fib* (200 bp) were observed with *Staph. aureus* templates, whereas fragments of *Se-fib* (700 bp) and *Se-124* (124 bp) were produced from *Staph. epidermidis* templates (Figure 1, left of marker). Each amplicon was verified by nucleotide sequencing. Combinations of primer pairs also confirmed the amplification of *23S rRNA*, *nuc*, and *Sa-fib* from *Staph. aureus* templates, and of *Se-fib* and *Se-124*

from *Staph. epidermidis* templates (Figure 1, right of marker).

Standardization of Multiplex PCR

The multiplex PCR was optimized to ensure that all the target gene sequences were amplified to a reasonably equivalent extent in a single reaction. However, amplification of *23S rRNA* was inhibited whenever primers for *FnBp* were also used in the same reaction mixture (Table 2). Hence, multiplex PCR was standardized with primers for *Sa-fib* (Figure 1), which could differentiate between *Staph. aureus* and *Staph. epidermidis* (see Table 3). In addition, the eubacterial *16S rRNA* primers inhibited the amplification of not only the species-specific *23S rRNA* gene but also that of *nuc* (Table 2, and data not shown). Therefore, the multiplex PCR included amplification of *23S rRNA*, *nuc*, *Sa-fib*, *Se-fib*, and *Se-124* fragments, whereas the amplification of eubacterial *16S rRNA* was performed in a separate tube.

Multiplex PCR was able to detect *Staph. aureus* or *Staph. epidermidis* in a mixture of the two. Fragments corresponding to *Staph. aureus* or *Staph. epidermidis* were amplified with the triplex or duplex primer pairs, respectively, and a mixture of the templates produced all 5 amplicons (Figure 1, right, and Figure 2, lanes 1–3). To verify the suitability of the test in the presence of other organisms, 2 different experiments were performed. First, each CNS and nonstaphylococcal species, as well as *Staph. aureus* and *Staph. epidermidis*, were cultured and template-DNA prepared separately, then an equal quantity of each template was mixed together. As expected, when templates from 6 different species of CNS and 5 different species of nonstaphylococci were spiked into the multiplex reaction, no inhibition of amplification of any of the 5 amplicons was observed (data not shown). However, as this does not approximate a mixed infection or culture, a second experiment was

Table 2. Standardization for selection of primers for multiplex PCR

Primer combination used							Products amplified						
23S ¹	Nuc	FnBp	Sa-fib	Se-fib	Se-124	E16S ²	23S	Nuc	FnBp	Sa-fib	Se-fib	Se-124	E16S
+							+						
+	+						+	+					
+		+					–		+				
	+	+					±	+	+				
+	+	+					+	+		+			
+	+		+				+	+		+	+		
+	+		+	+			+	+		+	+	+	
+	+		+	+	+	+	–	–		+	+	+	+

¹23S = *23S rRNA*.
²E16S = Eukaryotic *16S rRNA*.

Table 3. Bacterial strains used and the observed reactivity in PCR for various genes

Bacterial species (reference or isolate identification number)	Species-specific marker targeted					
	<i>Eu16S</i>	<i>23S</i>	<i>Sa-fib</i>	<i>Nuc</i>	<i>Se-124</i>	<i>Se-fib</i>
<i>Staphylococcus aureus</i> (MTCC96)	+	+	+	+	–	–
<i>Staphylococcus epidermidis</i> (MTCC3382)	+	–	–	–	+	+
<i>Staphylococcus epidermidis</i> (MTCC3615)	+	–	–	–	+	+
<i>Staphylococcus saprophyticus</i> (MTCC6155)	+	–	–	–	–	–
<i>Staphylococcus haemolyticus</i> (MTCC3383)	+	–	–	–	–	–
<i>Staphylococcus warneri</i> (MTCC3050)	+	–	–	–	–	–
<i>Staphylococcus sciuri</i> (MTCC6154)	+	–	–	–	–	–
<i>Staphylococcus xylosus</i> (MTCC7441)	+	–	–	–	–	–
<i>Staphylococcus chromogenes</i> (MTCC3545)	+	–	–	–	–	–
<i>Streptococcus agalactiae</i> ¹ (S55)	+	–	–	–	–	–
<i>Micrococcus luteus</i> (MTCC106)	+	–	–	–	–	–
<i>Escherichia coli</i> (MTCC739)	+	–	–	–	–	–
<i>Pseudomonas aeruginosa</i> (MTCC2453)	+	–	–	–	–	–
<i>Salmonella typhi</i> (MTCC743)	+	–	–	–	–	–

¹*S. agalactiae* was isolated from milk sample obtained from a clinical mastitis case.

performed where all CNS and nonstaphylococcal species were cultured in a single tube, with or without *Staph. aureus* and *Staph. epidermidis*, and DNA was extracted. Even under these circumstances, no inhibition of amplification was observed (Figure 2, lane 4). The absence of amplification with CNS and nonstaphylococcal species was not attributable to PCR inhibitors or to the inadequacy of the PCR protocol, because the control fragment of *16S rRNA* (240 bp) was amplified from all the bacterial species listed in Table 3, and the results for some of these are shown in Figure 2 (lanes 5–10). Further, the presence of the *16S rRNA* fragment in the DNA extracted from individual cultures of *Staphylococcus haemolyticus*, *Staph. saprophyticus*, *Staph. chromogenes*, *Escherichia coli*, *Salmonella typhi*, or *Streptococcus agalactiae* (Figure 2, lanes 5–10), or *Staphylococcus warneri*, *Staphylococcus sciuri*, or *Staphylococcus xylosus* (data not shown), indicated the presence of template DNA in the first set of experiments.

Application of Multiplex PCR to Field Isolates

To validate the multiplex PCR, the assay was applied to 602 bovine mastitis-associated isolates. These isolates were previously identified as belonging to the *Staphylococcus* genus and then to a particular species by culturing followed by various biochemical tests, such as sugar fermentation, coagulase activity on rabbit plasma, production of phosphatase, and thermonuclease activity (S. Sundareshan, unpublished data).

Whereas the biochemical method identified 242 isolates as *Staph. aureus* and 22 isolates as *Staph. epidermidis*, the respective number of isolates identified by multiplex PCR was 267 and 22 (Table 4). Therefore, the analytical specificity of multiplex PCR was 100%, meaning all the 242 isolates identified by phenotypic method were identified as *Staph. aureus* by multiplex

PCR. However, multiplex PCR could identify 25 CNS isolates as *Staph. aureus*. These results show that the multiplex PCR had diagnostic sensitivity and specificity of 100 and 93.5% for *Staph. aureus*, and 100 and 100% for *Staph. epidermidis*, respectively. The positive and negative predictive values were 90.64 and 100% for *Staph. aureus*, and 100 and 100% for *Staph. epidermidis*, respectively.

As the multiplex PCR was performed with overnight cultures, which yield abundant bacteria, it was important to compare the different fragments when amplified singly or in the multiplex reaction. The LOD of simplex and multiplex PCR assays were determined with DNA extracted from 10-fold serial dilutions of individual cultures of *Staph. aureus* and *Staph. epidermidis*. The detection limit of simplex PCR for *Staph. aureus* with *23S rRNA* was 10¹ cfu/mL, whereas it was 10² cfu/mL

Figure 2. Products amplified by simplex or multiplex PCR on mixed cultures of *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates were subjected to agarose gel electrophoresis. Lane 1 = *Staph. aureus*; lane 2 = *Staph. epidermidis*; lane 3 = *Staph. aureus* and *Staph. epidermidis*; lane 4 = mixed template of *Staph. aureus*, *Staph. epidermidis*, CNS, and nonstaphylococcus species listed in Table 3; lane M = DNA marker; lane 5 = *Staphylococcus haemolyticus*; lane 6 = *Staphylococcus saprophyticus*; lane 7 = *Staphylococcus chromogenes*; lane 8 = *Escherichia coli*; lane 9 = *Salmonella typhi*; and lane 10 = *Streptococcus agalactiae*.

Table 4. Comparison of conventional and PCR identification of staphylococci

Item	No.
Staphylococcal isolates tested	602
CNS	317
<i>Staph. epidermidis</i>	
Biochemical identification	22
PCR identification	22
<i>Staph. aureus</i>	
Biochemical identification	242
PCR identification	267

with *Sa-fib* and *nuc*. For *Staph. epidermidis*, the detection with *Se-124* and *Se-fib* was 10^1 and 10^2 cfu/mL, respectively. By contrast, the LOD of *Staph. aureus* and *Staph. epidermidis* with triplex (*23S rRNA*, *nuc*, *Sa-fib*) or duplex (*Se-fib*, *Se-124*) PCR, respectively, was 10^4 cfu/mL for each organism when the bacteria were grown overnight in mixed cultures. The detection limit dropped to 10^6 cfu/mL for simultaneous detection of *Staph. aureus* and *Staph. epidermidis* when all 5 sets of primers were used together (Table 5). To simulate conditions in milk, the LOD studies were repeated using cultures with known counts (cfu) or equivalent DNA diluted in pasteurized milk. The results did not differ from those described above (data not shown). We further investigated whether overnight enrichment culture would enhance the LOD. In these experiments, the analytical sensitivity was observed to be $2 \log_{10}$ better for duplex, triplex or multiplex reactions (Table 5). Thus, the multiplex PCR had an analytical sensitivity of 10^6 cfu/mL irrespective of whether buffer or pasteurized milk was used for dilution.

To explore whether the *fib* gene could be exploited to differentiate other staphylococci, sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) were aligned along with those of *Staph. aureus* and *Staph. epidermidis*. The only *fib* sequences available were those of *Staph. hemolyticus* and *Staph. saprophyticus*; indeed, primers unique to these organisms could be designed (Supplementary Figure S1). Accordingly, PCR would be predicted to amplify unambiguous fragments

of 350 and 620 bp for *Staph. hemolyticus* and *Staph. saprophyticus*, respectively, which are easily separable from fragments of *Staph. aureus* (210 bp) and *Staph. epidermidis* (720 bp) by agarose gel electrophoresis.

DISCUSSION

Several studies have highlighted the feasibility of using PCR for the detection of bacteria in milk from clinical and subclinical mastitis (Phuektes et al., 2001; Phuektes et al., 2003; Gillespie and Oliver, 2005; Taponen et al., 2009; Koskinen et al., 2010; Hirotaki et al., 2011; Shome et al., 2011). Because of the higher frequency of isolation (Thorberg et al., 2006; Oliveira et al., 2006; Shome et al., 2011), *Staph. aureus* and *Staph. epidermidis* have been the main organisms targeted for detection (Martineau et al., 1996; 1998; Edwards et al., 2001; Mason et al., 2001; Morot-Bizot et al., 2004; Taponen et al., 2009; Hirotaki et al., 2011; Shome et al., 2011). However, only a few studies have explored the simultaneous detection and differentiation of *Staph. aureus* and *Staph. epidermidis* but have targeted different genes in combination, or by using a single gene in real-time PCR assays (Morot-Bizot et al., 2004; Kilic and Basustaoglu, 2011; Shome et al., 2011; Chiang et al., 2012a,b). On the other hand, a few studies have explored the detection of multiple genes within a single species or a group of organisms (Mason et al., 2001; Tristan et al., 2003; Morot-Bizot et al., 2004; Cremonesi et al., 2005; Sabat et al., 2006; Jukes et al., 2010; Shome et al., 2011). As deletion in the target region of one gene can produce a false-negative result (Ghebremedhin et al., 2008; Horáková et al., 2008), we attempted a multiplex PCR to detect at least 2 genes in each of *Staph. aureus* and *Staph. epidermidis*. Our results show that multiplex PCR using *23S rRNA*, *nuc*, *fib*, and *Se-124* can be used for the identification and differentiation of *Staph. aureus* and *Staph. epidermidis*. One must note, however, that mastitis is a consequence of polymicrobial infection and colonization of the mammary gland, and our assay can only detect 2 of the causative agents.

Table 5. Limit of detection (LOD) of simplex and multiplex PCR using mixed culture of *Staph. aureus* and *Staph. epidermidis*

Gene fragment amplified					Count (cfu/mL) under indicated culture condition	
<i>23S</i>	<i>Nuc</i>	<i>Sa-fib</i>	<i>Se-124</i>	<i>Se-fib</i>	Nonenriched	Enriched
+	+	+	+	+	10^1	10^1
					10^2	10^2
					10^2	10^2
					10^1	10^1
					10^2	10^2
+	+	+	+	+	10^4	10^2
					10^4	10^2
					10^6	10^4
+	+	+	+	+		

The only genes that have been shown so far to differentiate *Staph. aureus* and *Staph. epidermidis* by themselves are *16S rRNA*, *nuc*, and *femA* (Edwards et al., 2001; Jukes et al., 2010; Hirotaki et al., 2011). Whereas differentiation with *16S rRNA* (Edwards et al., 2001) and *femA* (Jukes et al., 2010) was accomplished by melting curve analyses, differentiation with *nuc* (Hirotaki et al., 2011) was achieved with conventional PCR. In addition, studies with *16S rRNA* and *nuc* used isolates grown in culture, and did not conduct spiking studies, whereas those with *femA* applied the test to blood samples. Importantly, none of these groups performed LOD analyses. We have shown that sequences of *fib* can also be used to differentiate between the 2 species under a variety of scenarios, including mixed DNA, mixed culture, when spiked into milk, and when spiked and enriched. This is the first and the only colonization-associated gene described so far for the differentiation of *Staph. aureus* and *Staph. epidermidis*. In addition, it may also be possible to differentiate *Staph. hemolyticus* and *Staph. saprophyticus*, and the availability of complete gene sequences of other CNS may reveal the possibility of differentiating various other species as well.

One could argue that PCR which detects the mere presence of nucleic acids may not be useful to assess bacterial load in milk because even dead and degraded organisms could be detected. Indeed, it is possible that pasteurization will not eliminate DNA, and certain regions of the bacterial genome, particularly those that are thermoresistant, could be amplified by PCR even with a zero total plate count. The pasteurized milk that was used in this study was negative by simplex PCR for both *Staph. aureus* and *Staph. epidermidis* (data not shown). As the PCR could detect DNA equivalent to 10 to 100 cfu, it is safe to assume that remnants of DNA in pasteurized milk can be ruled out. It is, however, important to perform a quantitative analysis of the detectability of nucleic acid in comparison with cfu. This detailed and complicated analysis was beyond the scope of this study.

The multiplex PCR could identify *Staph. aureus* and *Staph. epidermidis* among a mixed culture containing more than a dozen staphylococci and nonstaphylococci. However, these experiments did not analyze competitive growth advantage of any of the organisms, if any exists. It is possible that certain organisms could grow better than others and therefore may reduce the LOD of targeted species. Nonetheless, even though the LOD was lower when mixed cultures were used, it was clear that the LOD was equivalent between *Staph. aureus* and *Staph. epidermidis*, suggesting equal growth under mixed culture conditions. The LOD by PCR for *Staph. aureus* or *Staph. epidermidis* was 10^4 cfu/mL, whereas

that for simultaneous detection of both the organisms was 100-fold lower (10^6 cfu/mL) when mixed templates or cultures were used. This could be attributed to PCR drift or competitive inhibition between primers (Lopes et al., 1999; Hsieh and Tsen, 2001; Kösters et al., 2002; Morot-Bizot et al., 2004). Under laboratory conditions, the LOD for staphylococci ranges from 10^0 to 10^5 cfu/mL. In various clinical specimens, including milk, the LOD ranges from 10^0 to 10^9 cfu/mL (Ramesh et al., 2002; Cremonesi et al., 2005; Gillespie and Oliver, 2005; Chiang et al., 2006; 2012a,b; Peles et al., 2007; Lee et al., 2008; Cressier and Bissonnette, 2011; Kilic and Basustaoglu, 2011; Shome et al., 2011). Raw milk obtained from apparently healthy cows has been shown to contain up to 10^7 cfu/mL of bacteria (Ramesh et al., 2002; Peles et al., 2007; Dan et al., 2008; Lingathurai and Vellathurai, 2010). It is conceivable that the bacterial load will be much higher, albeit variable, during mastitis. An extrapolation of the results of in vitro testing to milk would be difficult as the biology of the mammary gland and the physicochemical properties of the milk strongly influence microbial growth. In addition, pathogenic bacteria may outgrow nonpathogenic bacteria in vivo because of the virulence factors and invasive strategies utilized by the pathogens. Indeed, staphylococci can account for 50 to 70% of the microbial load of milk in cases of mastitis (Leonard and Markey, 2008; Lingathurai and Vellathurai, 2010; Mekibib et al., 2010). Thus, it may be possible to apply PCR not just for qualitative diagnosis, but also to evaluate the extent of the microbial load. In this context, advancements such as pit-stop multiplex PCR (Lopes et al., 1999) or real-time PCR could more accurately predict bacterial load once correlations are established.

In our study, 25 clinical isolates that were coagulase negative biochemically (and therefore classified as species other than *Staph. aureus*) were PCR positive for *Staph. aureus* markers. On further investigation, these isolates were found by PCR to carry at least a fragment of the coagulase gene (S. Sundareshan, unpublished data). This suggests the coagulase gene is not functional in these 25 isolates. A similar finding of mutated coagulase has been reported (Phonimdaeng et al., 1990). Together with the observations that proper identification of CNS species by phenotypic methods not only requires a combination of phenotypic and molecular assays (Akineden et al., 2011), one could argue that PCR-based assays are more accurate than biochemical assays.

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