Simultaneous Detection of Mastitis Pathogens, *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae* by Multiplex Real-Time Polymerase Chain Reaction

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ABSTRACT

The objective of this study was to develop a multiplex real-time polymerase chain reaction (PCR) method for simultaneous detection of *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis* directly from milk. A genetic marker specific for *Staph. aureus* was used for primers and dual-labeled probe design. The target for *Strep. agalactiae* primers and dual-labeled probe was selected from the *cfb* gene encoding the Christie-Atkins-Munch-Petersen factor. The plasminogen activator gene was the target for primers and dual-labeled probe design for *Strep. uberis*. Quarter milk samples ($n = 192$) were analyzed by the multiplex real-time PCR assay and conventional microbiological methods. An additional 57 quarter milk samples were analyzed in a separate real-time PCR assay for *Strep. agalactiae* only. Using an overnight enrichment step, the real-time PCR technique correctly identified 96.4% of all quarter milk samples; 91.7% of *Staph. aureus*, 98.2% of *Strep. agalactiae*, and 100% of *Strep. uberis*. Results of conventional microbiological methods were used to determine the sensitivity and specificity of the multiplex real-time PCR procedure. The sensitivity of the procedure to correctly identify *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from milk was 95.5%, and the specificity was 99.6%. Results of this study indicate that the multiplex real-time PCR procedure has the potential to be a valuable diagnostic technique for simultaneous identification of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from quarter milk samples.

(Key words: multiplex real-time polymerase chain reaction, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*)

Abbreviation key: ATCC = American Type Culture Collection, CAMP = Christie-Atkins-Munch-Petersen,

CPS = coagulase-positive *Staphylococcus*, C_T = cycle threshold.

INTRODUCTION

Worldwide, mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry. Several bacteria are implicated including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis* (Oliver et al., 1997). Identification of a bacterial pathogen in milk from a cow with mastitis is regarded as the definitive diagnosis of an IMI. Identification of mastitis pathogens is generally performed by traditional culture followed by biochemical tests on bacterial isolates (Oliver et al., 2004). Conventional microbiological methods have been the gold standard for identification of bacteria from milk. Identification of bacteria in most clinical laboratories is currently based on analysis of phenotypic characteristics utilizing biochemical tests, serotyping, and enzymatic profiles. Advantages associated with conventional culture methods are that viable bacteria can be identified as the causative agent of mastitis and antimicrobial susceptibilities can be performed providing information for selection of appropriate antimicrobial therapy. However, there are several disadvantages associated with current microbiological methods. A negative culture may result from residual antibiotics following antibiotic therapy or from low numbers of pathogens in the sample. Presence of leukocytes in milk from cases of clinical mastitis may also result in negative culture results (Phuektes et al., 2001). Current methods of mastitis pathogen identification are time consuming; identification of most pathogens by standard biochemical methods generally requires more than 48 h to complete. Inadequate pathogen detection or confirmation techniques have often delayed timely intervention in disease control intervention.

Use of DNA-based assays may circumvent some of the problems associated with conventional microbiological procedures. Perhaps the greatest single advantage of DNA-based diagnostic assays is that these

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methods focus on the unique nucleic acid composition of the bacterial genome rather than on phenotypic expression of products that nucleic acids encode. Therefore, DNA-based identification assays are subject to less variability compared with diagnostic methods based on phenotypic characterization. The DNA-based identification systems are targeted for specific pathogens, allow for rapid screening of a large number of pathogens simultaneously, and provide definitive confirmation of pathogens. Polymerase chain reaction protocols have been developed for identification of various mastitis pathogens (Jayarao et al., 1996; Forsman et al., 1997; Kim et al., 2001; Riffon et al., 2001; Daly et al., 2002; Meiri-Bendek et al., 2002; Phuektes et al., 2001, 2003). These PCR methods allow identification of bacteria within hours. With the use of real-time PCR, time for identification of bacteria directly from mastitis samples could be further reduced.

Real-time PCR utilizes the 5'-3' nuclease activity of *Taq* DNA polymerase to digest an internal fluorogenic probe labeled with a fluorescent reporter dye and a fluorescent quencher dye (Cai et al., 2003). During amplification, the probe is hydrolyzed relieving the quenching of the reporter dye, resulting in an increase in fluorescent intensity. This change in reporter dye fluorescence is quantitative for PCR product, and under appropriate conditions, for template. Such methods would be even more useful if they could be fine-tuned to simultaneously detect and quantify a mixture of pathogens in a sample. The objective of the present study was to develop a multiplex real-time PCR method to simultaneously detect common mastitis pathogens including *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from milk.

MATERIALS AND METHODS

Milk Samples

Quarter milk samples (n = 192) were collected from dairy cows from dairy research herds of The University of Tennessee. These samples were collected before milking using standard procedures described by the National Mastitis Council (Oliver et al., 2004). Before sample collection, teats of cows were dipped with a premilking teat disinfectant, cleaned thoroughly, and dried with individual disposable paper towels, and teat ends were sanitized with swabs containing 70% isopropyl alcohol. Milk samples were transported on ice, frozen, and maintained at -20°C until analysis. The University of Tennessee dairy research herds were free of *Strep. agalactiae*. Quarter milk samples (n = 57) previously identified as *Strep. agalactiae*-positive were obtained from Cornell University (Ithaca, NY). These *Strep. agalactiae*-positive samples were used in devel-

oping the real-time PCR assay for detection of *Strep. agalactiae*.

Conventional Microbiological Analysis

Milk samples were examined following procedures recommended by the National Mastitis Council (Hogan et al., 1999; Oliver et al., 2004). Briefly, 10 µL of foremilk was plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Becton Dickinson Co., Franklin Lakes, NJ). Plates were incubated at 37°C and bacterial growth was observed at 24-h intervals for 3 d. Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics, and catalase test. Isolates identified presumptively as staphylococci were tested for coagulase by the tube coagulase method, mannitol salt (Becton Dickinson), and DNase agar (Becton Dickinson). Isolates identified presumptively as streptococci were evaluated for growth in 6.5% NaCl, hydrolysis of esculin, and Christie, Atkins, and Munch-Petersen (CAMP)-reaction. Streptococcal organisms were identified to the species level using the API 20 Strep System (bioMérieux Inc., Hazelwood, MO). Gram-negative isolates were evaluated by their biochemical reactions on the following: MacConkey agar (Becton Dickinson), triple sugar iron agar (Becton Dickinson), urea agar (Becton Dickinson), oxidase (Becton Dickinson), motility, indole, and ornithine decarboxylase (Becton Dickinson), and identified to the species level using the API 20E System (bioMérieux Inc.).

Bacterial Strains

American Type Culture Collection (ATCC, Manassas, VA) reference strains used as positive controls for the multiplex real-time PCR assay included *Staph. aureus* (ATCC 10832), *Strep. agalactiae* (ATCC 27956), and *Strep. uberis* (ATCC 27958). Fifty-three different ATCC reference strains were used in the cross-reactivity study including: 11 *Staphylococcus* strains, 9 *Streptococcus* strains, 4 *Enterococcus* strains, 1 *Aerococcus* strain, 5 *Listeria* strains, 2 *Pseudomonas* strains, and 21 strains from the Enterobacteriaceae family (Table 1). An additional 25 *Strep. uberis* CAMP-positive strains were used for cross-reactivity with *Strep. agalactiae* specific primers and dual-labeled probe that targeted the *cfb* gene encoding the CAMP factor.

DNA Isolation

Bacterial DNA was extracted directly from reference strains and CAMP-positive *Strep. uberis* strains using

Table 1. Bacterial strains evaluated for specificity by multiplex real-time PCR for detection of *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis*.

Bacterial strain	Source/ ATCC ¹ no.	<i>Staph.</i> <i>aureus</i> - specific probe	<i>Strep.</i> <i>agalactiae</i> - specific probe	<i>Strep.</i> <i>uberis</i> - specific probe
<i>Staphylococcus aureus</i> ssp. <i>aureus</i>	10832	Positive	Negative	Negative
<i>Staphylococcus aureus</i> ssp. <i>aureus</i>	13709	Positive	Negative	Negative
<i>Staphylococcus aureus</i> ssp. <i>aureus</i>	29213	Positive	Negative	Negative
<i>Staphylococcus warneri</i>	27836	Negative	Negative	Negative
<i>Staphylococcus cohnii</i> ssp. <i>cohnii</i>	35622	Negative	Negative	Negative
<i>Staphylococcus hominis</i> ssp. <i>hominis</i>	27844	Negative	Negative	Negative
<i>Staphylococcus simulans</i>	27848	Negative	Negative	Negative
<i>Staphylococcus epidermidis</i>	14990	Negative	Negative	Negative
<i>Staphylococcus chromogenes</i>	43764	Negative	Negative	Negative
<i>Staphylococcus capitis</i> ssp. <i>capitis</i>	35661	Negative	Negative	Negative
<i>Staphylococcus xylosum</i>	35663	Negative	Negative	Negative
<i>Enterococcus faecalis</i>	27959	Negative	Negative	Negative
<i>Enterococcus faecalis</i>	29212	Negative	Negative	Negative
<i>Enterococcus faecium</i>	35667	Negative	Negative	Negative
<i>Enterococcus avium</i>	14025	Negative	Negative	Negative
<i>Aerococcus viridans</i>	11563	Negative	Negative	Negative
<i>Strep. dysgalactiae</i> ssp. <i>dysgalactiae</i>	27957	Negative	Negative	Negative
<i>Strep. dysgalactiae</i> ssp. <i>equisimilis</i>	35666	Negative	Negative	Negative
<i>Streptococcus salivarius</i>	13419	Negative	Negative	Negative
<i>Streptococcus mitis</i>	9811	Negative	Negative	Negative
<i>Streptococcus bovis</i>	27960	Negative	Negative	Negative
<i>Streptococcus agalactiae</i>	27956	Negative	Positive	Negative
<i>Streptococcus parauberis</i>	13386	Negative	Negative	Negative
<i>Streptococcus uberis</i>	13387	Negative	Negative	Positive
<i>Streptococcus uberis</i>	27958	Negative	Negative	Positive
<i>Listeria monocytogenes</i>	15313	Negative	Negative	Negative
<i>Listeria innocua</i>	33090	Negative	Negative	Negative
<i>Listeria welshimeri</i>	35897	Negative	Negative	Negative
<i>Listeria grayi</i>	25401	Negative	Negative	Negative
<i>Listeria ivanonii</i> ssp. <i>ivanonii</i>	19119	Negative	Negative	Negative
<i>Salmonella</i> Newport	6962	Negative	Negative	Negative
<i>Salmonella</i> Enteritidis	13076	Negative	Negative	Negative
<i>Salmonella</i> Typhimurium	14028	Negative	Negative	Negative
<i>Salmonella</i> Paratyphi A	11511	Negative	Negative	Negative
<i>Salmonella</i> Muenchen	8388	Negative	Negative	Negative
<i>Salmonella</i> Anatum	9270	Negative	Negative	Negative
<i>Salmonella</i> Montevideo	8387	Negative	Negative	Negative
<i>Salmonella</i> Choleraesuis	13312	Negative	Negative	Negative
<i>Salmonella</i> Worthington	9607	Negative	Negative	Negative
<i>Pseudomonas aeruginosa</i>	35554	Negative	Negative	Negative
<i>Pseudomonas aeruginosa</i>	35422	Negative	Negative	Negative
<i>Klebsiella pneumoniae</i>	12014	Negative	Negative	Negative
<i>Klebsiella pneumoniae</i>	13883	Negative	Negative	Negative
<i>Escherichia coli</i>	4350	Negative	Negative	Negative
<i>Escherichia coli</i>	31619	Negative	Negative	Negative
<i>Escherichia coli</i>	23545	Negative	Negative	Negative
<i>Escherichia coli</i>	25922	Negative	Negative	Negative
<i>Proteus vulgaris</i>	13315	Negative	Negative	Negative
<i>Proteus mirabilis</i>	25659	Negative	Negative	Negative
<i>Proteus mirabilis</i>	12453	Negative	Negative	Negative
<i>Enterobacter cloacae</i>	961	Negative	Negative	Negative
<i>Serratia marcescens</i>	13880	Negative	Negative	Negative
<i>Shigella flexneri</i>	12022	Negative	Negative	Negative
<i>Strep. uberis</i> , CAMP-positive ³ (n = 29)	UT ²	Negative	Negative	Positive

¹American Type Culture Collection.²University of Tennessee collection.³CAMP = Christie-Atkins-Munch-Petersen.

the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA). A loopful of bacterial cells was suspended in 200 μ L of PrepMan Ultra

sample preparation reagent in a 1.5-mL microcentrifuge tube. The sample was vortexed and heated for 10 min at 100°C. After incubation, the sample was

Table 2. Primers and dual-labeled probes for multiplex real-time PCR.

Name	Sequence	Length, bp	Fluorescent dye Reporter/Quencher
<i>Staphylococcus aureus</i> forward	TCAACGATATTCTTCACGACTAA	23	5'FAM/3'BHQ2 ¹
<i>Staph. aureus</i> reverse	CCAGCTTCGGTACTACTAAAG	21	
<i>Staph. aureus</i> probe	TCAAGACGGCTTTTACATACAGAACACA	28	
<i>Streptococcus agalactiae</i> forward	AGCTCTATTAGAAGTACATGCT	22	5'TexasRed/3'BHQ1 ²
<i>Strep. agalactiae</i> reverse	CATTTGCTGGGCTTGATTATT	21	
<i>Strep. agalactiae</i> probe	ATCAAGTGACAACCTCCACAAGTGGTAA	27	
<i>Streptococcus uberis</i> forward	AGAGGAATTCATCATGTTTAAACA	24	5'CY5/3'BHQ2 ³
<i>Strep. uberis</i> reverse	AATTGTAGAAGAACCATTGTGATGT	24	
<i>Strep. uberis</i> probe	AGCGTCTAACAACCTCGGCCTTTG	23	

¹5'FAM/3'BHQ2 = 6-Carboxyfluorescein / Black Hole Quencher 2.

²5'TexasRed/3'BHQ1 = Texas Red/ Black Hole Quencher 1.

³5'CY5/3'BHQ2 = Cyanine5/Black Hole Quencher 2.

centrifuged for 3 min at $16,000 \times g$. The supernatant was transferred to a new microcentrifuge tube, and 5 μ L was used as DNA template for the multiplex real-time PCR assay.

For isolation of bacterial DNA directly from milk, the method described by Allmann et al. (1995) was used with modifications as described by Hein et al. (2001). Milk samples (1 mL) were enriched with tryptic soy broth (1 mL, Becton Dickinson) and incubated at 37°C overnight. One milliliter of enriched sample was mixed with 130 μ L of digestion buffer (100 mM Tris, 100 mM EDTA, 0.5% SDS, pH 8.0) and digested with 100 μ L of pronase (10 mg/mL; Sigma-Aldrich, St. Louis, MO) at 40°C for 3 h. Bacterial cells were pelleted by centrifugation at $2500 \times g$ for 5 min at 4°C, and the fat layer and aqueous phase discarded. The pellet was washed 2 to 3 times with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Lysozyme (100 μ L, 2.0 mg/mL; Sigma-Aldrich) was added to the pellet and incubated for 15 min at room temperature (22°C). After incubation, 10 μ L of proteinase K (20 mg/mL; Roche Molecular Biochemicals, Indianapolis, IN) was added to each tube and incubated at 60°C for 45 to 60 min. The sample was vortexed and incubated at 95°C for 15 min, and then centrifuged at $16,000 \times g$ for 5 min at 4°C to remove cell debris. The supernatant was transferred to a new microcentrifuge tube, and 5 μ L was used as DNA template for the multiplex real-time PCR assay.

Primers and Dual-Labeled Probes

Primers and dual-labeled probes were designed using Beacon Designer 2.1 (Premier Biosoft International, Palo Alto, CA) and purchased from IDT (Coralville, IA). Primers and dual-labeled probes were developed for single real-time PCR assays and tested for specificity and sensitivity previously in this laboratory. Table 2 lists the sequence for each primer pair,

dual-labeled probe, and fluorescent dyes attached to dual-labeled probes. For detection of *Staph. aureus*, a genetic marker specific for *Staph. aureus* was designed based on primers used by Reischl et al. (2000) that amplified a 179-bp fragment within a *Staph. aureus*-specific genomic marker described previously by Martineau et al. (1998). Primers amplified a 160-bp fragment within the 179-bp fragment used by Reischl et al. (2000) and the dual-labeled probe hybridized to a 28-bp fragment within the 160-bp fragment. For *Strep. agalactiae*, the *cfb* gene encoding the CAMP factor was the target for *Strep. agalactiae* primers and probe (Ke et al., 2000). Primers were designed to amplify an 84-bp fragment within the 153-bp fragment designed by Ke et al. (2000), and the dual-labeled probe used in this study was a 27-bp fragment within the 84-bp fragment. For *Strep. uberis*, the plasminogen activator gene described by Sazonova et al. (2001) was the target. Primers were designed to amplify a 93-bp fragment of the plasminogen activator gene and the dual-labeled probe hybridized a 23-bp fragment within the 93-bp fragment.

Real-Time PCR Assay

The iQ Supermix (BioRad, Hercules, CA) was used for the multiplex real-time PCR assay. Each reaction contained 25 μ L of iQ Supermix (BioRad; 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 1.6 mM dNTP mix, 50 units/mL of iTaq DNA polymerase, and 6 mM MgCl₂), 2 μ L of 50 mM MgCl₂, 200 nmol of each primer and dual-labeled probe, and sterile water to bring the total volume to 50 μ L. The iCycler iQ real-time PCR detection system (BioRad) was programmed for 95°C for 2 min followed by 40 repeats of 95°C for 15 s and 57°C for 45 s. The fluorescence for each probe was measured during the 45-s hold at 57°C. For each sample, a cycle threshold (C_T) was calculated based on the baseline cycles and threshold value which is 10 times the mean

standard deviation of fluorescence in all wells over baseline cycles.

Detection Limit of Multiplex Real-Time PCR Assay from Pure Culture

Overnight cultures of *Staph. aureus* (ATCC 10832), *Strep. agalactiae* (ATCC 27956), and *Strep. uberis* (ATCC 27958) were used to prepare 10-fold serial dilution in UHT milk purchased from a local grocery store. Bacterial DNA was extracted directly from milk using modifications of the method described by Allmann et al. (1995), with modifications as described by Hein et al. (2001). The DNA from 10-fold dilutions was used as template for determining the sensitivity of the multiplex real-time PCR assay.

Enrichment vs. Nonenrichment of Milk Samples

Quarter milk samples ($n = 20$) were evaluated to determine if enrichment of milk samples was necessary for detection of pathogens in low numbers. For the enriched samples, 1 mL of trypticase soy broth (Becton Dickinson) was added to 1 mL of milk, mixed, and incubated overnight at 37°C. After incubation, 1 mL of enriched sample was used for isolation of bacterial DNA directly from milk using the method described by Allmann et al. (1995) with modifications as described by Hein et al. (2001). For the nonenriched sample, 1 mL of milk was used for bacterial DNA isolation as described previously for the enriched sample. Conventional bacterial methods were conducted on the 20 samples to identify bacteria and to determine the number of colony forming units per milliliter. Results were compared to determine the sensitivity of the real-time PCR assay to identify bacteria from milk samples of enriched vs. nonenriched samples.

Determination of Sensitivity and Specificity

Sensitivity and specificity of the multiplex real-time PCR was determined using the following formulas (Martin, 1984):

$$\text{Sensitivity \%} = \frac{\text{true positive samples}}{\text{true positive samples} + \text{false negative samples}} \times 100;$$

$$\text{Specificity \%} = \frac{\text{true negative samples}}{\text{true negative samples} + \text{false positive samples}} \times 100.$$

RESULTS

Specificity of PCR Primers and Probes

To check the specificity of primers and dual-labeled probes for real-time PCR analysis, DNA from pure

cultures of 53 type strains, and 25 *Strep. uberis* CAMP-positive strains was examined (Table 1). Only *Staph. aureus* strains tested positive in the real-time PCR assay with primers and probes specific for *Staph. aureus* and produced mean C_T values of 19.9 ± 0.9 . For primers and probe specific for *Strep. uberis*, only *Strep. uberis* strains were positive in the real-time PCR assay and produced mean C_T values of 11.3 ± 0.5 . Only *Strep. agalactiae* strains tested positive in the real-time PCR assay with primers and probes specific for *Strep. agalactiae* and produced mean C_T values of 16.1 ± 0.5 . Amplification of DNA from other bacterial strains evaluated either resulted in no fluorescence or a mean C_T value of >30 . Primers and probes were specific for the target organism with real-time PCR and did not show any cross-reactivity with other bacterial species.

Sensitivity and Detection Limits Using Pure Cultures

Overnight cultures of *Staph. aureus* (ATCC 10832), *Strep. agalactiae* (ATCC 27956), and *Strep. uberis* (ATCC 27958) were used to prepare 10-fold serial dilutions in UHT milk with bacterial concentrations ranging from 10^0 to 10^8 cfu/mL. Standard curves were constructed using mean C_T and bacterial cfu/mL. A linear relationship between C_T and log input DNA for each bacterium was observed. However, the minimum level of detection for *Staph. aureus* was 10^3 cfu/mL, and for *Strep. agalactiae* and *Strep. uberis*, it was 10^2 cfu/mL. After addition of an enrichment step, the minimum level of detection was lowered to 10^0 cfu/mL for *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis*.

Enrichment vs. Nonenrichment of Milk Samples

Twenty milk samples were evaluated with the real-time PCR method using enriched and nonenriched milk samples. Samples were chosen that contained *Staph. aureus* or *Strep. uberis*, or a mixture, with quantities ranging from 200 to $>10,000$ cfu/mL. Of the 20 milk samples, 10 were *Staph. aureus*-positive and 11 were *Strep. uberis*-positive; 1 milk sample was positive for both *Staph. aureus* and *Strep. uberis*. The real-time PCR assay using enriched milk samples was able to detect 9 of 10 *Staph. aureus* samples with a mean C_T value of 21.5, and 11 of 11 *Strep. uberis* samples with a mean C_T value of 19.3. Using nonenriched samples, the real-time PCR assay was only able to detect 5 of 10 *Staph. aureus* samples (mean C_T value of 32.1) and 10 of 11 *Strep. uberis* samples (mean C_T value of 30.3). The 1 *Staph. aureus* milk sample that was negative by both enrichment and nonenrichment was positive for *Staph. aureus* by real-time PCR when DNA

was isolated from the bacteria. This particular sample also contained *Strep. uberis*. Milk samples positive for *Staph. aureus* and *Strep. uberis* by conventional methods, but negative with real-time PCR using nonenriched milk contained 400 to 10,000 cfu/mL of *Staph. aureus* or *Strep. uberis*. Results indicate that enrichment is needed to detect low numbers of bacteria in milk. Enrichment may also be necessary to dilute inhibitory substances present in milk because the detection limit for this multiplex real-time PCR using nonenriched milk samples was 10^3 cfu/mL for *Staph. aureus* and 10^2 cfu/mL for *Strep. uberis*.

Quarter Milk Samples

A single real-time PCR assay for the *Strep. agalactiae* *cfb* gene was performed on 57 quarter milk samples previously identified as *Strep. agalactiae*-positive by conventional methods. The single real-time PCR correctly identified 98.2% (56/57) quarter milk samples positive for *Strep. agalactiae*. The 57 quarter milk samples were used to develop the *Strep. agalactiae* real-time PCR assay before developing the multiplex real-time assay for *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis*. The remaining milk sample was not of sufficient quantity to be used in evaluation of the multiplex real-time PCR assay.

Quarter milk samples ($n = 192$) screened previously by conventional microbiological methods were evaluated by the multiplex real-time PCR method for detection of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis*. The multiplex real-time PCR analysis, including enrichment and DNA purification, was repeated twice for each quarter milk sample. This assay correctly identified 96.4% (185/192) of all quarter milk samples (Table 3). The multiplex real-time PCR correctly identified 91.7% of *Staph. aureus*, and 100% of *Strep. uberis*. Using the formula described by Martin (1984), the sensitivity of this procedure to correctly identify *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from milk was 95.5%, and the specificity was 99.6%.

This assay detected the target pathogen even though the sample contained more than one type of bacteria. Forty-four (Table 3) milk samples contained 2 different bacteria. The multiplex real-time PCR assay was able to identify all (15/15) milk samples that contained *Strep. uberis* and other bacteria including CNS, *Staph. aureus*, *Strep. dysgalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia liquefaciens* (Table 3). The multiplex real-time PCR assay was able to identify 10 of 13 milk samples that contained *Staph. aureus* and other bacteria including CNS, *Corynebacterium*

bovis, *Streptomyces* spp., *Enterococcus faecalis*, and *Strep. dysgalactiae* (Table 3).

The assay was able to differentiate *Staph. aureus* from other coagulase-positive *Staphylococcus* (CPS) species. Five isolates identified as CPS by conventional microbiological methods (coagulase-positive, mannitol-negative, and DNase-negative) were negative by real-time PCR of the bacterial isolate for *Staph. aureus* and by the multiplex real-time PCR directly from milk. Two *Staph. aureus* milk samples from mastitis quarters were negative for *Staph. aureus* on repeated occasions by multiplex real-time PCR. However, analysis of the DNA extracted from the isolated bacteria alone produced positive results.

DISCUSSION

The assay described in the present study is the first to use a multiplex real-time PCR format for detection of pathogens directly from milk. Phuektes et al. (2001, 2003) developed a multiplex PCR for detection of *Staph. aureus*, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* that targeted 16S to 23S rRNA spacer regions. Sensitivity of the multiplex PCR was lower than for each individual PCR assay, and enrichment was needed to bring the threshold for detection by multiplex PCR for all bacteria assayed to 1 cfu/mL. Although this assay was able to detect 4 different bacteria, there are only 6 base-pair differences between the *Strep. agalactiae* and *Strep. dysgalactiae* ssp. *dysgalactiae* amplified fragments, which would make interpreting results difficult. Use of the multiplex real-time PCR eliminates the need for post-PCR analysis by gel electrophoresis.

Numerous methods for isolating bacterial DNA directly from milk have been reported in the literature and involve a wide variety of substances including Chelex-100 (Kim et al., 2001), spin columns (Phuektes et al., 2001, 2003; Riffon et al., 2001), lysozyme and proteinase K (Meiri-Bendek et al., 2002), diatomaceous earth (Martinez et al., 2001), alkaline extraction (Daly et al., 2002), and pronase (Allmann et al., 1995; Hein et al., 2001). Several methods were evaluated in this study for isolating bacteria directly from milk. Methods that use proprietary reagents such as InstaGene Matrix (BioRad) and PrepMan Ultra reagent (Applied Biosystems) were evaluated. Although these reagents were faster and more convenient than other methods evaluated, results were not consistent and reproducible when trying to isolate bacterial DNA from milk. The method first described by Allmann et al. (1995) and modified by Hein et al. (2001) was the most consistent and reproducible method evaluated for isolation of bacteria directly from whole milk sam-

Table 3. Results of 192 quarter milk samples evaluated by the multiplex real-time PCR for detection of *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis*.

		<i>Staphylococcus aureus</i>		<i>Streptococcus uberis</i>		<i>Streptococcus agalactiae</i>	
		Positive	Negative	Positive	Negative	Positive	Negative
CNS	26	1 ¹	25	0	26	0	26
CNS/ <i>Strep. uberis</i>	7	0	7	7	0	0	7
<i>Staph. aureus</i> /CNS	4	3	1	0	4	0	4
CNS/ <i>Strep. dysgalactiae</i>	2	0	2	0	2	0	2
CPS ²	5	0	5	0	5	0	5
<i>Staph. aureus</i>	59	56	3	0	59	0	59
<i>Staph. aureus</i> /other bacteria ³	6	6	0	0	6	0	6
<i>Staph. aureus</i> / <i>Strep. uberis</i>	3	1	2	3	0	0	3
<i>Strep. dysgalactiae</i> /other bacteria ⁴	17	0	17	0	17	0	17
Negative ⁵	10	0	10	0	10	0	10
<i>Strep. uberis</i> / <i>Strep. dysgalactiae</i>	2	0	2	2	0	0	2
<i>Strep. uberis</i>	48	0	48	48	0	0	48
<i>Strep. uberis</i> /GNR ⁶	3	0	3	3	0	0	3
Total	192	67	125	63	129	0	192

¹CNS positive for *Staph. aureus*, C_T = 20.6.

²CPS = Coagulase-positive, mannitol-negative, DNase-negative *Staphylococcus*.

³*Corynebacterium bovis*, *Streptomyces* spp., *Enterococcus faecalis*, and *Streptococcus dysgalactiae*.

⁴*Streptococcus bovis*, *Enterococcus* spp., and *Lactococcus* spp.

⁵No growth.

⁶GNR = Gram-negative rod; *Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia liquefaciens*.

ples. Casein, a major protein in milk, is composed of several similar proteins along with calcium and phosphorus that forms a casein micelle. Pronase from *Streptomyces griseus* has the ability to break down virtually all proteins, including casein, into their individual amino acids. Breaking down casein and the casein micelle with pronase would allow better access to the bacteria for lysis by lysozyme and proteinase K. This method, although more time consuming than other methods, gave the most consistent and reproducible results.

Other methods to overcome PCR inhibitors present in milk involve dilution of the sample. An enrichment step was added to overcome PCR inhibition and to increase the sensitivity of the assay. With this added step, sufficient bacteria were present to allow detection of as few as 1 cfu/mL. The higher sensitivity may be due to dilution of inhibitory substances in the enrichment broth and the increased number of organisms. The presence of PCR inhibitors in milk and a variety of other clinical samples such as urine, blood, and feces have been reported (Higuchi, 1989; Toye et al., 1998). Little is known about inhibitory components present in milk. In the present study, 2 milk samples that contained *Staph. aureus* were repeatedly negative for *Staph. aureus* by multiplex real-time PCR; however, DNA extracted from the bacteria isolated from the milk was positive for *Staph. aureus* using the same *Staph. aureus*-specific primers and probe (data not shown). Both of these samples were from mammary quarters with clinical mastitis, suggesting the pres-

ence of a unique PCR inhibitor. These findings suggest that PCR inhibitors were present in the original sample, but not removed by either the DNA isolation procedure or the enrichment step.

Addition of an enrichment step has been reported and appears to be necessary for detecting low numbers of bacteria (<1000 cfu/mL). Meiri-Bendek et al. (2002) developed a PCR method for detection of *Strep. agalactiae* in milk that targeted the conserved areas within 16S rRNA. The sensitivity of this assay increased from between 10⁴ and 10⁵ to 1 cfu/mL after overnight selective enrichment. Phuektes et al. (2001) needed enrichment in a multiplex PCR for detection of *Staph. aureus*, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* to detect levels of 1 cfu/mL.

The multiplex real-time PCR assay described here used information concerning primers for *Staph. aureus* and *Strep. agalactiae* that were shown to be sensitive and specific. Primers and probes for *Staph. aureus* were designed within the region used by Reischl et al. (2000). Reischl et al. (2000) and Martineau et al. (1998) reported 100% sensitivity and specificity for this particular *Staph. aureus* target sequence. Primers used in this study amplified a 160-bp fragment and the dual-labeled probe hybridized to a 28-bp fragment within the 160-bp fragment. The *cfb* gene encoding the CAMP factor was used as the genetic target for real-time PCR for detection of *Strep. agalactiae*. Ke et al. (2000) designed oligonucleotides after sequence comparison of the *cfb* genes from *Strep. agalactiae*, *Strep. uberis*, and *Strep. pyogenes*, which re-

vealed that the 3 genes were moderately divergent. Ke et al. (2000) evaluated 125 bacterial and fungal species and 162 strains of *Strep. agalactiae*. Only *Strep. agalactiae* strains produced an increase in fluorescence signal that indicated a positive result for *Strep. agalactiae*. Primers in this study were designed to amplify an 84-bp fragment within the 153-bp fragment designed by Ke et al. (2000), and the dual-labeled probe used in this study is a 27-bp fragment within the 84-bp fragment. For *Strep. uberis*, primers were designed to amplify a 93-bp fragment of the plasminogen activator gene and the dual-labeled probe hybridized to a 23-bp fragment within the 93-bp fragment. This is the first report of use of the plasminogen activator gene of *Strep. uberis* as a target for PCR reaction.

CONCLUSIONS

The multiplex real-time PCR assay for simultaneous detection of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from milk described herein was developed to evaluate the potential use of multiplex real-time PCR in the diagnosis of mastitis. By utilizing an overnight enrichment step for the multiplex real-time PCR assay, the minimum level of detection was 10^0 cfu/mL for *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis*. The multiplex real-time PCR takes slightly less than 24 h to complete compared with 36 to 48 h for identification by standard culture methods. The multiplex real-time PCR technique correctly identified 96.4% of all quarter milk samples, and correctly identified 91.7% of *Staph. aureus*, 98% of *Strep. agalactiae*, and 100% of *Strep. uberis*. The sensitivity of this procedure to correctly identify *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from milk was 95.5% and the specificity was 99.6%. To increase the sensitivity of this assay for detection of *Staph. aureus*, further research is needed to identify and overcome the effects of PCR inhibitors from milk on the multiplex real-time PCR. Results of this study indicate that the multiplex real-time PCR procedure has the potential to be developed as an alternative diagnostic method for the simultaneous identification of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* directly from quarter milk samples.

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