Development of a loop-mediated isothermal amplification assay for the detection of *Streptococcus agalactiae* in bovine milk

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ABSTRACT

Streptococcus agalactiae is a well-characterized bovine mastitis pathogen that is known to be highly contagious and capable of spreading rapidly in affected dairy herds. Loop-mediated isothermal amplification (LAMP) is a novel molecular diagnostic method that has the capability to provide rapid, cost-effective screening for pathogens to support on-farm disease control and eradication programs. In the current study, a LAMP test was developed to detect *S. agalactiae* in milk. The assay was validated on a bank of existing clinical mastitis milk samples that had previously been identified as *S. agalactiae* positive via traditional microbiological culture techniques and PCR. The LAMP assay was conducted on bacterial colonies and DNA extracted from milk in tube- and plate-based formats using multiple detection platforms. The 1-h assay conducted at 64°C exhibited repeatability (coefficient of variation) of 2.07% (tube) and 8.3% (plate), sensitivity to ~20 pg of extracted DNA/reaction, and specificity against a panel of known bacterial mastitis pathogens. Of the 109 known *S. agalactiae* isolates assessed by LAMP directly from bacterial cells in culture, 108 were identified as positive, in accordance with PCR analysis. The LAMP analysis from the corresponding milk samples indicated that 104 of these milks exhibited a positive amplification curve. Although exhibiting some limitations, this assay provides an opportunity for rapid screening of milk samples to facilitate on-farm management of this pathogen.

Key words: bovine, loop-mediated isothermal amplification, mastitis, milk, *Streptococcus agalactiae*

INTRODUCTION

Streptococcus agalactiae is a contagious mastitis pathogen capable of causing clinical and subclinical mastitis in cattle and it can have a substantial financial impact on dairy producers due to its effects on milk

quality and quantity (Keefe, 2012). The prevalence of herds infected with *S. agalactiae* varies worldwide. Although some countries have apparently managed to almost eradicate the pathogen (Bradley et al., 2007; Piepers et al., 2007), others, such as Egypt, Canada, Estonia, and Germany, report herd prevalences of 20, 9, 12, and 29%, respectively (Tenhagen et al., 2006; Kalmus et al., 2011; Francoz et al., 2012; Elhaig and Selim, 2015). Australian reports suggest that *S. agalactiae* is present in from 1 to 6% of herds in southeastern Australia (Penry et al., 2014). The herd prevalence in Scandinavia is increasing; in Denmark, for example, the herd prevalence has increased from 3% in 2003 to 6.1% in 2009 (Mweu et al., 2012a).

The primary reservoir of *S. agalactiae* is infected udders and the organism has the potential to spread rapidly in herds with poorly functioning milking equipment or unhygienic milking practices (Keefe, 1997). Disease eradication has been possible with the implementation of control programs such as outlined in the 5-point plan for control of contagious mastitis pathogens that revolves around hygienic management practices including post-milking teat disinfection and regular milking system analysis and maintenance, as well as prompt treatment of infected animals, dry cow therapy, and the culling of chronically infected cows (Edmondson, 2011; Green and Bradley, 2013). The rapid and accurate identification of infected animals is pivotal to the success of such disease control programs (Gurjar et al., 2012).

Microbiological culture methods are routinely used to identify cows infected with *S. agalactiae* (Hogan et al., 1999). This includes growth on tryptose soy agar plates containing 5% defibrinated sheep blood of organisms with typical colony morphology (small, 1 to 2 mm in diameter, moist, convex and translucent colonies; most displaying β hemolysis with fewer displaying α or no hemolysis) after incubation for 24 to 48 h. Confirmation of genus and species is made via Gram staining (cocci morphology with positive Gram staining) and characteristic biochemical test results—negative for catalase production, positive on the Christie, Atkins, Munch-Petersen (CAMP) test, positive on the trehalose test, and negative on the esculin hydrolysis test

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(Hogan et al., 1999). However, microbiological culture is time consuming and laborious (Gurjar et al., 2012). In addition, there is potential for misclassification of *S. agalactiae* as another *Streptococcus* species (Edmondson, 2011).

Analysis of isolates and milk samples utilizing molecular methods such as PCR are described in the literature (Phuektes et al., 2001; Meiri-Bendek et al., 2002; McDonald et al., 2005; Paradis et al., 2012), and PCR testing for *S. agalactiae* is now widely commercially available. The PathoProof Mastitis PCR Assay (Thermo Fisher Scientific, Vintaa, Finland) is a commercially available multiplex real-time test kit that simultaneously detects the presence of several different bacterial species in milk (Koskinen et al., 2010), one of which is *S. agalactiae*, and which is purported to have the advantage over microbiological culture in that it is more sensitive and specific, particularly with its ability to identify bacteria that are no longer viable or whose growth is inhibited (Taponen et al., 2009; Koskinen et al., 2010; Mweu et al., 2012b).

Loop-mediated isothermal amplification (LAMP) technology is another molecular method of nucleic acid amplification that was first described in 2000 (Notomi et al., 2000) and has since been applied to the detection of pathogenic bacteria, viruses, and parasites in milk, feces, blood, mucus, and dust (Okamura et al., 2008; Mori and Notomi, 2009; Wang et al., 2010). The main advantage of this technology is that, like PCR, it is purported to be faster, less expensive, and highly specific for the target sequence. Its potential main advantage over PCR is that, in theory, because it amplifies DNA under isothermal conditions, it could be implemented in a field setting, requiring only a water bath or heat block for the reaction to occur (Mori and Notomi, 2009) and with relatively minimal sample preparation. Thus, the aim of the current study was to utilize LAMP technology to develop an assay for the specific detection of *S. agalactiae* in milk to provide a rapid and cost-effective detection system compatible with automation and application in herd testing laboratories.

MATERIALS AND METHODS

Composite Milk Samples Used in Development of PCR and LAMP Assays

In 2010 and 2011, *S. agalactiae* was isolated from 120 milk samples that were routinely submitted to the Livestock Veterinary Teaching and Research unit milk quality laboratory, Faculty of Veterinary Science at the University of Sydney, using standard milk culture techniques (Hogan et al., 1999). The samples were obtained

from 3 different dairy farms, one each from New South Wales, Victoria, and Queensland. The milk samples were then frozen at -80°C until analyzed.

Isolation and Storage of *S. agalactiae* for PCR and LAMP Assays

On commencement of the study, the milk samples previously identified as positive for *S. agalactiae* were thawed and recultured on tryptose soy agar plates containing 5% defibrinated sheep blood (SBA, Micromedia, Victoria, Australia) and incubated for 24 h at 37°C for growth of *S. agalactiae* colonies. Isolates were identified as *S. agalactiae* if they had the characteristic colony morphology (small, 1 to 2 mm in diameter, moist, convex, and translucent colonies on blood agar; most displaying β hemolysis), were gram-positive cocci, and had the characteristic biochemical test results (negative for catalase production, positive in the CAMP test, positive for trehalose fermentation, and negative for esculin hydrolysis; Hogan et al., 1999). Single colonies were inoculated into brain heart infusion broth with a sterile loop and incubated overnight in a shaking incubator (Edwards Group Pty Ltd., Narellan, NSW, Australia) at 37°C . Glycerol was then added to the broth to a final concentration of 16% (vol/vol) and this was then stored at -80°C .

Extraction of Whole Bacterial Cell and Genomic *S. agalactiae* DNA

A loop scraping of *S. agalactiae* colonies from each sample on SBA was then incubated at 37°C in 50 mL of DNA-quality water for up to 4 h to obtain whole bacterial cell lysate and then either analyzed immediately by PCR or LAMP or frozen at -20°C until analysis at a later date. Genomic DNA was purified from selected samples using a column-based method for gram-positive bacteria (DNeasy Blood and Tissue Kit, Qiagen, Doncaster, VIC, Australia). Briefly, after overnight growth in 3 mL of brain heart infusion broth, isolates were harvested by centrifugation at $5,000 \times g$ for 10 min. Isolates were then resuspended in lysis buffer containing 0.5 mg of proteinase K (supplied with the kit) and incubated at 56°C for 30 min. Following incubation, 200 μL of buffer (supplied in the kit) and 200 μL of 100% ethanol were added to the samples, which were vortexed thoroughly before applying to the spin column. After centrifugation at $6,000 \times g$ for 1 min, the flow-through was discarded and 2 wash steps were performed. The membrane containing the DNA was then dried by centrifuging for 3 min at $20,000 \times g$. The DNA was then eluted in 100 μL of elution buffer.

Milk Lysate Preparation for LAMP Analysis

In an effort to minimize costs associated with routine testing, milk lysates were prepared for analysis via LAMP by a technique modified from that previously reported (Meiri-Bendek et al., 2002). Briefly, 1 mL of milk was placed into a 1.5-mL microfuge tube and centrifuged at $20,000 \times g$ for 2 min in a microfuge (model Eppendorf 5424, Eppendorf South Pacific, North Ryde, NSW, Australia). Following this, the milk fat was removed from the surface of the sample with a sterile plastic loop and a sterile cotton swab and the supernatant was discarded. The sediment pellet was resuspended in $1 \times$ Tris-EDTA, pH 8.0, and centrifuged at $20,000 \times g$ for 2 min. This step was repeated as required until the supernatant appeared clear. The sediment was resuspended in a PCR master mix $1 \times$ reaction buffer (Thermopol, New England BioLabs, Arundel, QLD, Australia) and lysozyme (1 $\mu\text{g}/\mu\text{L}$, from chicken egg white; Sigma, Castle Hill, NSW, Australia) and incubated for 20 min at room temperature. Following this, proteinase K (5 μL of 20 mg/mL; Qiagen) was added to the suspension, which was then vortexed and incubated for 1 h at 56°C , followed by a further 15-min incubation at 100°C . The solution was then centrifuged for 1 min at $20,000 \times g$. The supernatant containing the milk lysate was harvested from the pellet and analyzed immediately by LAMP or stored at -20°C for later analysis.

Establishment of PCR

A PCR assay based on a published PCR diagnostic test (Meiri-Bendek et al., 2002) was used to confirm the identity of the isolate as *S. agalactiae* and to make a comparative analysis of the LAMP assay. Briefly, this included a *S. agalactiae*-specific PCR assay as well as a general *Streptococcus* spp. PCR assay that was used as a confirmatory positive control. The amplification occurred in a 10- μL volume consisting of PCR buffer (5 $\times$ Colorless GoTaq; Promega, Madison, WI), 2.5 mM MgCl_2 , 2.5 mM deoxynucleotide triphosphates (dNTP), 2.5 pmol of each of the forward and reverse primers, 0.25 U of DNA polymerase (GoTaq DNA Polymerase, Promega), 3 μL of DNA template, and DNA-quality water to volume. The amplification was carried out using a PCR thermocycler (GeneAmp PCR systems model 9700, Perkin Elmer Applied Biosystems, Waltham, MA) as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and 72°C for 7 min at the end of the reaction. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel containing 535 $\mu\text{g}/\text{mL}$ ethidium bromide and a 100-

bp ladder molecular weight marker (concentration 130 ng/ μL , 2 μL per lane; Promega). The expected size of PCR products for *S. agalactiae* was 120 bp and for *Streptococcus* spp. PCR assay was 207 bp, and these were confirmed by the gel electrophoresis. The PCR products for both the *S. agalactiae*-specific PCR and the *Streptococcus* spp. PCR assay were sequenced by a service provider [Australian Genome Research Facility (AGRF), St Lucia, QLD, Australia] using the forward and reverse primer utilizing standard cycle sequencing protocols

S. agalactiae and Cytochrome *b* LAMP Primer Design

A minimum of 3 different primers sets were designed for both the 16S rRNA gene of *S. agalactiae* and the bovine Cytochrome *b* gene using Primer Explorer V4 (<http://primerexplorer.jp/e>; Eiken Chemical Company Ltd., Tokyo, Japan). The optimal primer set was chosen based on pilot experiments that evaluated performance with respect to level of amplification and initial sensitivity of primer sets at standard reaction conditions. Each primer set consisted of 6 primers: 2 outer (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and 2 loops (loop F and loop B). The FIP and BIP primer sets for the *S. agalactiae* LAMP were purified by HPLC (GeneWorks, Hindmarsh, SA, Australia). The primer sequences for each assay are listed in Table 1. The first primer was based on unique *S. agalactiae* DNA sequences within the 16S subunit of the rRNA gene (NR_040821). The second primer set was prepared from a sequence within the bovine mitochondrial cytochrome *b* gene (D34635.1) and was included as a positive control to confirm the extraction of amplifiable DNA from the milk sample. If the result of the cytochrome *b* assay for a particular sample was negative, then the DNA extraction process for that sample was repeated.

S. agalactiae and Cytochrome *b* LAMP Reactions

The LAMP reactions were performed within PCR tubes (Scientific Specialties Inc., Lodi, CA) in a Loopamp real-time turbidimeter (model LA-320C, Eiken Chemical Co.) in 24 μL of a mixture composed of 0.8 M betaine; 40 pmol of FIP/BIP, 5 pmol of F3/B3, and 20 pmol of LF/LB primers per reaction; $1 \times$ detergent-free PCR master mix (6 mM MgSO_4 ; 1.4 mM of each dNTP; 8 U of *Bst* polymerase; Thermopol, New England BioLabs, Arundel, Queensland, Australia); 0.25 μL of 10% Tween 20; 8.25 μL of DNA quality water; and 1 μL of DNA template as milk lysate. Water (1 μL)

Table 1. Primer sequences used in the *Streptococcus agalactiae* and cytochrome *b* loop-mediated isothermal amplification analyses

Primer name ¹	Sequence (5' to 3')
<i>S. agalactiae</i> 16S rRNA	
FIP	5'-GGCCGATCACCCTCTCAGGTGTTGGTGAGGTAAAGGCTCA-3'
BIP	5'-GAGACACGGCCAGACTCCTGTTGCTCGGTCAGACTTCC-3'
F3	5'-TGTGAGATGGACCTGCGT-3'
B3	5'-AACCTTCTTCACTCACGCG-3'
LF	5'-CGGCTATGTATCGTCGCCTTGG-3'
LB	5'-GCAGCAGTAGGGAATCTTCGG-3'
Bovine cytochrome-b	
FIP	5'-ATCACTCGGGTTTGATGTGAGGAGACCCAGATAACTACACCC-3'
BIP	5'-TCCCCAACAACTAGGAGGAGTTTGGAGGTGTGTAGTAGGG-3'
F3	5'-GTACTATTGCGACCCGAC-3'
B3	5'-TGAGTGGTCGGAATATTATGC-3'
LF	5'-TGTGTTGAGTGGATTGGCT-3'
LB	5'-TAGCCCTAGCCTTCTCTATCC-3'

¹Each primer set consisted of 6 primers: 2 outer (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and 2 loops (L; LF and LB).

was used instead of DNA template as a no-template control and a milk sample previously determined to be negative for *S. agalactiae* by microbiological culture was used as a negative milk control sample in each analysis. *Streptococcus agalactiae* genomic DNA (2 ng/ μ L) and a milk sample determined to be positive for *S. agalactiae* via microbiological culture were used as positive controls for each analysis. The reaction was carried out at 64°C for 45 min for *S. agalactiae* and 60 min for cytochrome *b* followed by 2 min at 80°C to terminate the reaction. Although a specific time cut-off was not used, a sample was considered as positive if turbidity was clearly observed before any nonspecific turbidity was observed in the no-template control samples. Real-time turbidity curve characteristics were also used to determine validity of observations.

S. agalactiae LAMP Assay Specificity, Repeatability, and Sensitivity

The LAMP primers were examined for their specificity using *S. agalactiae* and 12 other bacterial species commonly found in milk samples (Table 2). Genomic DNA was extracted from these bacterial species (DNeasy Blood and Tissue Kit, Qiagen) by the same method as described above for genomic *S. agalactiae* and samples were analyzed in triplicate.

A single representative sample of *S. agalactiae* genomic DNA (2 ng/reaction) was analyzed 8 times to determine the repeatability of the *S. agalactiae* LAMP assay and measured by the end-point optical density (OD) or the turbidity threshold (the time when an OD of 0.1 was reached).

Table 2. Specificity analysis of the *Streptococcus agalactiae* loop-mediated isothermal amplification assay using absorbance at 650 nm demonstrating amplification of *S. agalactiae* only

Bovine mastitis pathogen	Species of origin	Source ¹	Specificity (amplification) ²
<i>Streptococcus agalactiae</i>	Bovine	LVT&RU	+
<i>Staphylococcus aureus</i>	Bovine	ATCC 25923	—
<i>Streptococcus uberis</i>	Bovine	LVT&RU	—
<i>Streptococcus dysgalactiae</i>	Bovine	LVT&RU	—
<i>Enterococcus faecalis</i>	Bovine	LVT&RU	—
<i>Mycoplasma bovis</i>	Bovine	LVT&RU	—
<i>Mycoplasma californicum</i>	Bovine	LVT&RU	—
<i>Escherichia coli</i>	Bovine	LVT&RU	—
<i>Klebsiella pneumoniae</i>	Not listed	VPBCC	—
<i>Pseudomonas aeruginosa</i> VPB 591	Buffalo	VPBCC	—
<i>Listeria monocytogenes</i> VPB 43	Not listed	VPBCC	—
<i>Pasteurella multocida</i> VPB 906S	Fowl	VPBCC	—
<i>Nocardia asteroides</i> VPB 4	Dog	VPBCC	—

¹LVT&RU = Livestock Veterinary Teaching and Research Unit, University of Sydney (Sydney, Australia); ATCC = American Type Culture Collection (Manassas, VA); VPBCC = Veterinary Pathology and Bacteriology Culture Collection, University of Sydney.

²Where + indicates amplification and — indicates no amplification.

To ascertain the sensitivity of the *S. agalactiae* LAMP assay, serial 10-fold dilutions (20 ng/reaction to 2 pg/reaction) of extracted genomic *S. agalactiae* DNA in Tris-EDTA were analyzed in the LAMP assay in triplicate.

LAMP Analysis via a 96-Well Plate Format

To increase potential throughput, a 96-well plate format was developed and a comparative analysis was performed with the tube format described above. Briefly, for the plate format, LAMP reactions were performed in 96-well round-bottomed polypropylene plates (cat. no. 650201; Greiner Bio-one, Frickenhausen, Germany) in a hybridization oven (Sigma) in 35 µL of a mixture composed of 0.8 M betaine; 56 pmol of FIP/BIP, 7 pmol of F3/B3, and 28 pmol of LF/LB primers per reaction; 1× detergent-free PCR master mix (6.25 mM MgSO₄, 1.4 mM of each dNTP; 11 U of *Bst* polymerase; Thermopol, New England BioLabs); 0.35 µL of 10% Tween 20; 10.15 µL of DNA-quality water; and 1.4 µL of DNA template (genomic DNA 2 ng/µl or milk lysate). Positive and negative control samples were included as per the tube assay. Before placing in the hybridization oven set at 64°C, 96-well plates were sealed with plate sealers (ThermalSeal RT2RR, Excel Scientific, Victorville, CA), and a prewarmed metal block was placed on top of the plate to limit evaporation and condensation on the plate sealer. Upon completion of the reaction (45 min for *S. agalactiae* and 60 min for cytochrome *b*), the plate was removed from the oven and placed on a cool pack before removal of the metal block. Several detection systems were assessed in the 96-well plate format including turbidity at 420 and 650 nm on a plate reader (POLARstar Optima, BMG LabTech, Ortenberg, Germany) and hydroxy naphthol blue dye (HNB; Fluka,

Sigma) read at 650 nm after removal of the plate sealer. A stock solution of HNB at a concentration of 3 mM in distilled water was prepared and added to each well at a final concentration of 120 µM added to the reaction mix before amplification. A color change of the HNB from purple to blue indicates a positive reaction. The HNB method proved the most sensitive and reproducible. Sensitivity, specificity, and repeatability of the plate assay were analyzed as for the tube format of the assay as described above.

RESULTS

Culturing of S. agalactiae from Milk Samples

Of the 120 samples from which *S. agalactiae* had previously been isolated, 109 could be successfully recultured, resulting in the isolation of *S. agalactiae* based on colony morphology and biochemical testing.

PCR Analysis of S. agalactiae Whole-Cell DNA Obtained from Milk Isolates and Milk Lysates

Of the 109 bacterial isolates that were successfully isolated from the milk samples on reculturing and identified as *S. agalactiae* by colony morphology and biochemical testing, 108 were PCR positive on the general *Streptococcus* spp. PCR assay (Table 3). The same 108 DNA samples were also positive on the *S. agalactiae*-specific PCR assay (Table 3). Sequence analyses confirmed the identity of the PCR products.

There was no amplification of *S. agalactiae* DNA in PCR reactions that were conducted on milk lysates (using the same sample from which the isolates were purified) in pilot experiments and therefore we did not attempt PCR analysis of all 120 milk samples.

Table 3. Analysis by PCR and loop-mediated isothermal amplification (LAMP) of bacterial whole-cell DNA and lysates of milk samples for *Streptococcus agalactiae*

Assay ¹	Bacterial whole-cell DNA	Milk sample lysate
PCR		
<i>Streptococcus</i> spp.	108/109	No amplification in pilot study ²
<i>Streptococcus agalactiae</i>	108/109	
LAMP ³		
Bovine cytochrome <i>b</i>	108/109	119/120
<i>Streptococcus agalactiae</i>	108/109	104/120

¹A *Streptococcus* species general control was included in the PCR analysis; a bovine cytochrome *b* DNA control was included for the LAMP analysis to confirm quality of DNA extraction.

²The PCR reactions that were conducted on the whole bacterial cells did not amplify *S. agalactiae* DNA on milk lysates in pilot experiments, and therefore analysis of the entire milk lysate sample set was not conducted.

³For the LAMP analysis, all samples were run as a single sample in the first run. Samples were repeated twice if a negative result was obtained. If a negative result was obtained twice, samples were re-extracted and re-analyzed.

LAMP of Whole-Cell *S. agalactiae* DNA

Of the 109 bacterial isolates that were successfully isolated from the milk samples on reculturing, 108 were positive for *S. agalactiae* on LAMP analysis (Table 3), a result consistent with the results obtained in the PCR assays. The single isolate that was negative on PCR analysis was also negative on LAMP analysis.

S. agalactiae and Cytochrome *b* LAMP on Milk Lysates Extracted from Individual Milk Samples

The results of the LAMP analysis on individual milk sample lysates are presented in Table 3. Of the 120 milk samples from which *S. agalactiae* was originally cultured, all but one were positive on LAMP analysis for bovine cytochrome *b*. If a negative result was obtained twice, samples were re-extracted and reanalyzed. The cytochrome *b* gene could be detected to a dilution of 1:10,000 of milk lysate. Of the 120 milk samples from which *S. agalactiae* was originally cultured, 104 lysates of these milk samples were positive for *S. agalactiae* DNA on LAMP analysis after testing in triplicate. Samples were repeated twice if a negative result was obtained, which resulted in 76 out of 120 being positive on the first run; an additional 20 were positive after the second run and an additional 11 were positive following the third run. Of the 104 milk sample lysates positive for *S. agalactiae* DNA on LAMP analysis, 9 were originally culture positive but a *S. agalactiae* isolate could not be recovered from them on reculturing after freezing.

Specificity, Repeatability, and Sensitivity of *S. agalactiae* LAMP Assay

To determine the specificity of the *S. agalactiae* LAMP assay, 13 known bovine mastitis pathogens were analyzed in the assay in triplicate. The assay amplified only *S. agalactiae* DNA (Table 2).

The repeatability of the *S. agalactiae* LAMP assay was determined by analyzing a single representative sample of *S. agalactiae* genomic DNA 8 times added to the reaction at 2 ng/reaction. For the end-point OD, the coefficient of variation (CV) was ~2.07%. An alternative method of analysis was performed by measuring the turbidity threshold value (the time when an OD of 0.1 was reached); for this alternate analysis, the CV was 1.39%.

Serial 10-fold dilutions (20 ng to 2 pg/reaction) of the extracted genomic DNA of *S. agalactiae* in Tris-EDTA were subjected to LAMP analysis, resulting in detection of DNA at concentrations as low as 20 pg/reaction.

Table 4. The comparative performance of the *Streptococcus agalactiae* loop-mediated isothermal amplification (LAMP) assay in tube and plate formats

Item	Tube	Plate
Temperature	64°C	64°C
Time	1 h	1 h
CV ¹ (end point)	2.07%	8.30%
Sensitivity (gDNA dilution) ²	20 pg/reaction	20 pg/reaction ³
Specificity ⁴	<i>S. agalactiae</i> only	<i>S. agalactiae</i> only

¹The coefficient of variation (CV) was determined for the end-point optical density by analyzing a single sample of *S. agalactiae* genomic DNA (2 ng/reaction) 8 times.

²To ascertain the sensitivity of the *S. agalactiae* LAMP assay, serial 10-fold dilutions (20 ng to 2 pg/reaction) of extracted genomic *S. agalactiae* DNA (gDNA) in Tris-EDTA were analyzed in the LAMP assay in triplicate.

³Two out of three replicates were detectable at 20 pg/reaction.

⁴To determine specificity, 13 known bovine mastitis samples (see Table 2) were analyzed in triplicate.

Comparative LAMP Analysis of a 96-Well Plate Versus the Tube Format

The comparative performance of the LAMP assay in terms of repeatability, sensitivity, and specificity in the 96-well plate and tube formats is outlined in Table 4. Sensitivity and specificity were similar between the 2 formats. Only 2 out of 3 replicates were detectable at 20 pg of genomic DNA per reaction in the plate format compared with 3 out of 3 for the tube format. All 3 replicates were detected at 200 pg per reaction for both tube and plate formats. There was greater variation in repeatability with the plate format (CV = 8.30%) compared with the tube format (CV = 2.07%).

DISCUSSION

The current study describes the successful development of a LAMP assay for the detection of *S. agalactiae* DNA in milk samples by both tube and 96-well plate formats, with comparable sensitivity and specificity to conventional PCR assays used in this study and other studies described in the literature (Martinez et al., 2001; Meiri-Bendek et al., 2002).

The detection of *S. agalactiae*-infected cows is fundamental to dairy biosecurity and disease control programs (Keefe, 2012). Rapid detection is highly desirable due to the highly contagious nature of the pathogen and the potential for disease spread in the interim between sample collection and the delivery of test results. Testing protocols, typically directed at individuals and groups of animals initially to detect infected cows and subsequently to verify segregation and quarantine procedures, have been effective at establishing and maintaining a disease-free population (Keefe, 2012).

In this study, when LAMP analysis was compared with PCR analysis for whole-cell DNA, the results were identical. However, the PCR protocol that was conducted on whole-cell DNA in the present study did not amplify *S. agalactiae* DNA when used on milk lysates in pilot experiments and therefore, a comparative analysis (i.e., PCR versus LAMP) of the entire milk lysate sample set was not conducted. The finding that a PCR reaction was unable to amplify specific DNA in milk lysates is supported by a previous study showing that LAMP is less sensitive to inhibitory substances present in biological samples compared with PCR (Kaneko et al., 2007). In the current study, the LAMP method enabled amplification of DNA in less purified milk lysate samples without the need for initial microbiological culture. With the simplified sample preparation protocol used in this study, results could be obtained in less than 6 h, which is considerably less than the 48 h or so required for microbiological culture and biochemical testing or subsequent speciation via PCR for confirmation of identity.

Of the 120 samples previously identified as positive for *S. agalactiae* and then stored at -20°C for later analysis, *S. agalactiae* could not be recultured from 11 frozen milk samples. The freeze-thawing process may explain the lack of success in culturing these milk samples. Several studies have evaluated the effects of freezing on viability of mastitis pathogens, with some studies reporting no change in culture results (Dinmore et al., 1992; Murdough et al., 1996), whereas others reported an increase (Schukken et al., 1989) or decrease in culture of *S. agalactiae* following freezing (Luedecke et al., 1972; Bashandy and Heider, 1979; Sol et al., 2002). Despite the fact that one of the latter reports (Sol et al., 2002) found a slight decrease in growth of *S. agalactiae* in frozen samples compared with the same sample not subjected to freezing, it was not considered significant enough to deter the authors from still recommending freezing and culture for the isolation of major udder pathogens. In each of these studies, the duration of freezing was short, with 10 wk being the maximum time frame investigated by one study (Luedecke et al., 1972). Some of the samples in the current study may have been frozen for up to 12 mo and may also have been freeze-thawed on previous occasions before culture for this study. Such repeated freeze-thaw and extended freezing may have affected the culture viability of some samples. Irrespective of the reason for the lack of success in culturing these samples, 9 of these 11 samples could be identified as positive for *S. agalactiae* DNA on LAMP analysis of milk lysates, indicating that the LAMP assay was able to identify positive samples that could not be identified on microbiological culture following freezing of the

sample. Similarly, though not specifically tested for in the present study, LAMP technology could potentially detect samples where antimicrobial therapy inhibits microbiological growth.

The number of milk lysate samples returning a positive result on the LAMP assay increased with increasing repetitions of the assay (i.e., 76 out of 120 being positive on the first run, an additional 20 being positive after the second run, and an additional 11 being positive following the third run). The analysis of the replicates for each sample was conducted in parallel rather than simultaneously. The main reason for this was to ensure similar conditions with each run, as a large number of samples had to be analyzed within the constraint of the 32-tube format of the real-time turbidity meter. Although LAMP was more successful at amplifying DNA from the milk lysate samples than PCR (which could not amplify DNA in any of the samples in the pilot study), there were still a significant number of false-negative observations. Thus, it would be advisable to run samples in at least duplicate to attain satisfactory sensitivity of the assay. The level of observed false-negative observations might also be reduced by further refinement of the milk lysate preparation procedure or development of an alternative LAMP primer set for the detection of the 16S rRNA gene of *S. agalactiae*. By comparison, however, it should be noted that the bovine cytochrome *b* assay could detect DNA at a 1/10,000 dilution of milk lysate and resulted in very few false-positive results, with only 1 sample out of 120 failing to amplify bovine cytochrome *b* DNA in the present study. Although we could not specifically measure it, concentrations of bovine DNA within the cell lysates were likely significantly higher than the concentration of DNA from *S. agalactiae*. Similarly, as *S. agalactiae* is an extracellular pathogen, it was difficult to determine if the cell lysate preparation method, particularly the centrifugation step, may have differentially altered the relative proportions of bovine and bacterial DNA.

One of the challenges of the LAMP assay development was the design of the primers, with at least 3 sets required for the initial validation for both the *S. agalactiae* and cytochrome *b* assays. Others have reported evaluating multiple primer sets to optimize the assay (Foord et al., 2012). Furthermore, other researchers have attempted to enhance primer design, particularly for the FIP and BIP primers, to improve the design process (Kimura et al., 2011). In the current study, primer design was critical to the development of a successful assay, with factors such as the guanine-cytosine (GC) content being vitally important considerations (Notomi et al., 2000). During the current study, primer stability (particularly of the FIP and BIP primers) was a potential constraint, with some of the synthesized

primer sets remaining functional, whereas others seemingly degraded quickly and affecting assay performance (data not shown).

The ability to easily validate LAMP assays is a confounding issue in assay development. Typical validation for PCR assays may include estimation and characterization of PCR product by agarose gel electrophoresis or other means to confirm specificity of amplification. This may also be supported by sequencing of the PCR product. Similar electrophoresis and sequencing of LAMP products is either difficult or not achievable in the LAMP assay due to the complexity of the loop-mediated amplification process, with the specificity of the assay being reliant on the complementarity of the multiple primer sets involved in the LAMP amplification process.

Given that, in theory, the LAMP method could be implemented in a field setting, requiring only the appropriate primers, a DNA polymerase, and a water bath or heat block for the reaction to occur, it may represent a cheaper diagnostic option compared with PCR, where costly equipment for amplification and detection are required. For field analysis, an alternative detection system to real-time monitoring with the turbidimeter would need to be used, such as visualization of turbidity or HNB dye. The LAMP reaction produces insoluble magnesium pyrophosphate during the amplification reaction, which is visible as turbidity in the reaction solution (Mori and Notomi, 2009). This production of magnesium pyrophosphate can also be exploited in enhanced detection systems such as the use of HNB, with the color of the HNB being dependent upon the pH of the solution or changes in Mg^{2+} ion concentration (Goto et al., 2009).

The 96-well format may offer further potential for minimizing costs with its enhanced throughput, low setup and analysis costs, and flexibility in detection platforms, which may also allow for automation. Importantly, the assay performance assessed for the tube and plate assays was similar for both formats (Table 4). As with field assays utilizing tubes or HNB dye, the main disadvantage of the 96-well format as an endpoint assay is the loss of the valuable real-time information in terms of amplification curves available on the real-time turbidity meter. Amplification curve shape, take off, and turbidity threshold point are all valuable for interpretation of the data and to discount false-positive amplification. The type of plate used in the plate format experiments was crucial, with polypropylene U-bottomed plates enabling the optimal results (data not shown). Other studies report polypropylene plates as being the most suitable and suggest that polystyrene plates resulted in nonuniform heat conduction among the wells of the plate (Goto et al., 2009). The heating

and cooling regimen required so that condensation does not form on the plate sealer films is also an important consideration compared with the use of tubes. The formation of condensation on the plate sealer seriously affected the ability of the spectrophotometer to read the OD value of the sample in the wells underneath and so it was vital to eliminate this. In the current study, a prewarmed metal block was used; however, other studies report success with using a prewarmed (to 63°C) polyethylene gel sheet on the top cover (Goto et al., 2009). The use of HNB dye in the 96-well format was able to provide a visual confirmation for data produced on the plate reader.

The current study demonstrates that there may be some utility in the application of LAMP for the detection of mastitis pathogens. The advantages of the LAMP technique over PCR—specifically the ability to amplify DNA from less purified samples (milk lysates compared with genomic DNA extraction from milk for PCR), the variety of simple detection methodologies (visual turbidity or HNB dye color change), short amplification times, and the requirement for only basic equipment due to the isothermal nature of the LAMP amplification process—warrant further consideration and development of this technique to support rapid in-field mastitis pathogen detection techniques.

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