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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 (coccoid morphology with positive Gram staining) and characteristic biochemical test results—negative for catalase production, positive on the Christie, Atkins,

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Munch-Petersen (CAMP) test, positive on the trehalose test, and negative on the esculin hydrolysis test (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 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 kept 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 × *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 × *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 × *g*. The DNA was then eluted in 100 μL of elution buffer.