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Rapid detection of *Streptococcus uberis* in raw milk by loop-mediated isothermal amplification

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ABSTRACT

A loop-mediated isothermal amplification (LAMP) method to detect *Streptococcus uberis* in raw milk was developed and evaluated. Three genes (*sodA*, *pauA*, *cpn60*) were assessed for their suitability as targets in LAMP. The analytical sensitivity was 120, 120, and 12 fg per assay for the *sodA*, *pauA*, and *cpn60* assays, respectively, with a detectable signal within 8 min for the highest concentration (12 ng/assay) and ~60 min for the lowest concentrations. The LAMP assays correctly identified 7 *Strep. uberis* strains among a set of 83 mastitis pathogens. To enable DNA isolation from raw milk, a new method was used in which a pretreatment with a cocktail of lysing enzymes was performed before an established procedure. This method resulted in an analytical sensitivity of 48 cfu/assay for the *sodA* LAMP assay using raw milk spiked with *Strep. uberis*, corresponding to 2.4×10^4 cfu/mL milk. For raw milk samples from cows experimentally infected with *Strep. uberis*, results of enumeration were largely reflected by results of LAMP. Evaluation of the *sodA* LAMP assay with 100 raw milk field samples, of which 50 were *Strep. uberis* culture-negative and 50 *Strep. uberis* culture-positive, showed that the assay had a diagnostic sensitivity of 96.0% and a diagnostic specificity of 96.0%. In conclusion, the described LAMP assay may offer a simple alternative for convenient and sensitive detection of *S. uberis* in raw milk, provided a compatible rapid DNA isolation procedure is available.

Key words: loop-mediated isothermal amplification (LAMP), *Streptococcus uberis*, mastitis, raw milk

INTRODUCTION

Mastitis is a common and costly infectious disease affecting dairy farms (Hogeveen et al., 2011) and one of the major reasons for use of antibiotics in dairy farming (Pieterse and Todorov, 2010). Several bacteria are implicated as causative agents, including *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and coliforms such as *Escherichia coli* (Reyher et al., 2012). Monitoring of udder health is most frequently done by SCC and bacteriological culturing of milk (Lam et al., 2009). After culturing, additional typing techniques are required to determine the causative agent. Early detection and identification of pathogens could accelerate decisions on treatment, thereby contributing to animal health and reduced use of antibiotics (Trevisi et al., 2014).

To improve pathogen detection, various molecular diagnostic tests have been developed for identification of mastitis pathogens directly in milk (Koskinen et al., 2009), but rapid and sensitive tests with the potential for on-farm usage are not yet commercially available. Loop-mediated isothermal amplification (**LAMP**) is an alternative method of nucleic acid amplification to PCR that holds great promise for rapid on-farm diagnostics. The LAMP method is faster than PCR and less demanding in terms of the quality of the template DNA; moreover, an expensive dedicated machine is not required (Kaneko et al., 2007; Lucchi et al., 2010; Njiru et al., 2012).

For LAMP, at least 4 primers and a DNA polymerase with strand displacement activity are required (Notomi et al., 2000). Additional loop primers can accelerate the reaction but are not essential (Nagamine et al., 2002). Various methods of detection are possible, including the formation of optic visible magnesium pyrophosphate (turbidity) or fluorescence by DNA-intercalating dyes such as EvaGreen (Goto et al., 2007; Tomlinson et al., 2010; Bekele et al., 2011).

Although LAMP assays have been described for mastitis pathogens such as *Staph. aureus* and *Strep.*

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agalactiae (Zhao et al., 2013a), so far, LAMP has not been used for *Streptococcus uberis*, an important environmental pathogen accounting for a significant proportion of subclinical and clinical IMI (Bradley et al., 2007; Zadoks et al., 2011). To investigate the possibilities of LAMP for rapid on-farm diagnostics of mastitis pathogens, we developed a combined DNA isolation method and a LAMP assay for detection of *Strep. uberis* in raw milk. Assay performance was evaluated with raw milk obtained from experimentally infected animals, as well as with a selection of raw milk samples from the field, of which half were bacteriologically positive for *Strep. uberis.* The combination of the DNA isolation method and a LAMP assay targeting the *Strep. uberis sodA* gene allowed for rapid detection of *Strep. uberis* in raw milk, with outcomes that compared well with results of bacteriological methods.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains of various bacterial species isolated from milk of cows with mastitis (Table 1) were selected from in-house strain collections. Bacteria were grown on sheep blood agar heart infusion (**HIS**) plates (Central Veterinary Institute, Lelystad, the Netherlands) for 18 h at 37°C under aerobic conditions. The species of each isolate was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF MS**; MALDI Biotyper 3.1, Bruker Daltonics GmbH, Leipzig, Germany).

For DNA isolation, bacteria were harvested by scraping from the surface of a plate grown to confluence. The bacteria were suspended in 1 mL of PBS (0.1 *M* NaCl, 33 m*M* Na_2HPO_4, 17 m*M* $KH_2PO_4{\cdot}2H_2O$; pH 7.4) and pelleted by centrifugation at 10,000 × *g* for 15 min. The pellets were stored at −20°C until further use.

From the selected set of bacteria, one *Strep. uberis* strain (strain 2.28) was chosen as a positive control. This isolate was cultured on sheep blood agar HIS plates for 18 h at 37°C. From one colony, an overnight culture (18 h) was prepared in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, UK) at 37°C under aerobic conditions. A 1:100 dilution in Todd-Hewitt broth was grown to exponential phase (optical density of 0.5 at 600 nm). The number of colony-forming units per milliliter of the liquid culture was determined by serial dilution and plating on HIS agar. Subsequently, bacteria were pelleted by centrifugation at 10,000 × *g* for 10 min, and the pellet was resuspended in Nutrient Broth Medium No. 2 (Central Veterinary Institute) with 15% glycerol to a concentration of 1.9×10^9 cfu/mL. Aliquots of 1 mL were frozen at −80°C until further use.

For the experimental infection of cows, *Strep. uberis* strain O140J (Leigh et al., 1990) was used. This strain was grown on Columbia agar blood base plates (Oxoid Ltd.) containing 6% (vol/vol) horse blood and 0.1% (vol/vol) esculin for 18 h at 37°C. Liquid cultures of *Strep. uberis* O140J were grown in Todd-Hewitt broth (Oxoid Ltd.) for 16 h at 37°C. Subsequently, cultures were diluted 1:10 in Todd-Hewitt broth and grown to exponential phase to prepare the inoculum.

Isolation of DNA from Bacterial Cultures and Raw Milk

Aliquots of 200 μL of suspended bacteria containing 0.4×10^9 cfu (see above) or 200 μL of milk were incubated for 30 min at 37°C with 50 μL of Tris-EDTA buffer [20 mmol/L Tris-HCl (pH 8.0), 2 mmol/L EDTA] containing achromopeptidase, lysostaphin, lysozyme, and mutanolysin (1,000 U/mL, 20 μg/mL, 1 mg/mL, and 100 U/mL, respectively; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). Subsequently, the mix was incubated for 1 h at 56°C with 20 μL of proteinase K in 180 μL of ATL buffer from the DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands), and bacterial DNA was isolated according to the procedure of the manufacturer and taken up in 200 μL of water. Concentrations of DNA were determined by using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA).

Table 1. Bacterial strains (n = 83), originating from cows with mastitis, that were used as reference material (all strains were from an in-house strain collection)

Gram status	Species	No. of strains
Gram-positive	*Staphylococcus aureus*	3
	Staphylococcus chromogenes	8
	Staphylococcus epidermidis	5
	Staphylococcus haemolyticus	2
	Staphylococcus hyicus	4
	Staphylococcus simulans	3
	Staphylococcus warneri	4
	Streptococcus agalactiae	7
	Streptococcus castoreus	2
	Streptococcus dysgalactiae	13
	Streptococcus uberis	7
	Corynebacterium bovis	1
	Enterococcus faecalis	2
	Listeria monocytogenes	2
	Trueperella pyogenes	1
Gram-negative	*Serratia marcescens*	3
	Escherichia coli	7
	Klebsiella oxytoca	3
	Klebsiella pneumoniae	3
	Salmonella spp.	3