NOTE *Bacteriology*

Development of a Loop-Mediated Isothermal Amplification Method for Detecting *Streptococcus equi* subsp. *zooepidemicus* and Analysis of Its Use with Three Simple Methods of Extracting DNA from Equine Respiratory Tract Specimens

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(Received 17 March 2014/Accepted 9 May 2014/Published online in J-STAGE 28 May 2014)

ABSTRACT. *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is a dominant pathogenic bacterium in equine pneumonia. We developed a specific loop-mediated isothermal amplification (LAMP) method, which targets the gene encoding sorbitol-6-phosphate 2-dehydrogenase (*sorD*), for detecting *S. zooepidemicus* and examined the clinical efficacies of its use in combination with each of 3 DNA extraction methods easily used by veterinary practitioners, namely the Loopamp PURE DNA Extraction Kit, InstaGene Matrix and a conventional boiling method. The LAMP method plus the Loopamp PURE DNA Extraction Kit gave higher rates of positivity than the other combinations in both clinical and spiked samples containing clinically significant concentrations (>1 × 10^4 CFU/ml) of *S. zooepidemicus*.

KEY WORDS: DNA extraction method, equine, LAMP method, Loopamp PURE DNA Extraction Kit, *Streptococcus equi* subsp. *zooepidemicus*

doi: 10.1292/jvms.14-0140; *J. Vet. Med. Sci.* 76(9): 1271–1275, 2014

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) is a beta-hemolytic Gram-positive Lancefield group C bacterium found in a wide range of species, including horses, pigs, monkeys, dogs and humans [7, 21, 23, 25]. It is part of the normal bacterial microflora of the upper respiratory tract and caudal reproductive tract of horses [25]. *S. zooepidemicus* is an opportunistic pathogen associated with a wide variety of diseases in horses, including pneumonia, mastitis, placentitis and endometritis [5, 10, 13, 14]. In particular, *S. zooepidemicus* is a predominant pathogen in the bacterial pneumonia resulting from transport of horses for long periods [19]; the mortality rate from pleuropneumonia secondary to this type of bacterial pneumonia is high [22, 24]. Rapid diagnosis and appropriate antimicrobial use by veterinary practitioners are therefore important in preventing the progression of *S. zooepidemicus*-related disease.

Multiplex PCR [1] and multiplex real-time PCR [4] for detecting *S. zooepidemicus* and *Streptococcus equi* subsp. *equi* (*S. equi*) have been described previously. These 2 published PCR-based methods use 2 genes: *sodA*, which encodes a manganese-dependent superoxide dismutase, for detecting both *S. zooepidemicus* and *S. equi*; and *seeI*, which encodes pyrogenic mitogen SePE-I, for detecting only *S. equi*. The 2 methods cannot judge the presence or absence of *S. zooepidemicus* when a sample contains *S. equi*, because they do not use a primer set that can detect only *S. zooepidemicus*. Therefore, the 2 methods are appropriate for identifying each species in pure culture, but are not suitable for detecting *S. zooepidemicus* in clinical samples. Carbohydrate (e.g. lactose, sorbitol and trehalose) fermentation testing is commonly used to differentiate between *S. zooepidemicus* and *S. equi* [2]. All *S. equi* strains lack the *sorD* gene that encodes sorbitol-6-phosphate 2-dehydrogenase and therefore lack the ability to ferment sorbitol, whereas all *S. zooepidemicus* strains possess *sorD* [9]. This genetic basis indicates that *sorD* should be suitable for differentiating *S. zooepidemicus* from *S. equi.*

The loop-mediated isothermal amplification (LAMP) method was developed as a new type of nucleic acid amplification method [18]. LAMP amplifies nucleic acids with high speed, specificity and efficiency, and it can be performed under isothermal conditions with no special equipment. In recent years, LAMP has been applied clinically as a method for rapid detection of various pathogens [8, 17]. Here, we therefore developed a novel LAMP method specific to *S. zooepidemicus* and targeting *sorD*.

The LAMP primers for detecting *S. zooepidemicus* were designed on the basis of published sequences of *sorD* by using PrimerExplorer V4 software (Fujitsu Limited, Tokyo, Japan). The GenBank accession numbers of *sorD* used for designing the primers were NC011134, CP002904 and FM204884. The primer set included 5 primers: 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP) and 1 loop primer (Loop B). The primer sequences for the LAMP method are shown in Table 1. The reaction mixture was prepared by using a Loopamp DNA Amplification Kit (Eiken Chemical Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's instructions. In brief, 25 μl of reaction mixture was prepared to contain 12.5 μl of 2 × reaction mix buf-

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Table 1. LAMP primer set used to detect *Streptococcus zooepidemicus*

Primer	Sequence (5′→3′)	Gene location (bp) [a)]
F3	ATGGCCTCTGAGGCAGG	427–443
B3	TCTGGTCAACGGTTTTTCCT	627–646
FIP [b)]	GCCCAAGAGCGTGTATAGCTGTGAAGGCTCAGAAGGGCAAAG	497–518 (F1c) − 448–467 (F2)
BIP [c)]	GGCAAGCATGGCGTTCGAGTCTTCATAAGCCAATGTCCGCA	529–548 (B1c) −587–607 (B2)
Loop B	ACCAGGTATCATGGAGGCGAC	561–581

a) Positions of LAMP primers from the start codon of the *S. zooepidemicus sorD* gene (Accession number: NC011134). b) FIP primer consists of F1c and F2 regions. c) BIP primer consists of B1c and B2 regions.

Table 2. Bacterial strains used in this study

Bacterial species	Number of strains
Streptococcus equi subsp. *zooepidemicus*	51
Streptococcus equi subsp. *equi*	50
Streptococcus dysgalactiae subsp. *equisimilis*	23
Streptococcus bovis	4
Streptococcus pneumoniae	4
Actinobacillus equuli	3
Escherichia coli	3
Klebsiella pneumoniae	3
Pasteurella pneumotropica	3
Pseudomonas aeruginosa	3
Rhodococcus equi	3
Salmonella enterica subsp. *enterica* serovar Abortusequi	3
Salmonella enterica subsp. *enterica* serovar Typhimurium	3
Staphylococcus aureus	3
Staphylococcus hyicus	3
Streptococcus mitis	3
Streptococcus acidominimus	2
Streptococcus equinus	2
Streptococcus mutans	2
Streptococcus constellatus	1

fer, 0.2 μM of each outer primer (F3 and B3), 1.6 μM of each inner primer (FIP and BIP), 0.8 μM of loop primer (Loop B), 1.0 μl of Bst DNA polymerase (8 units/μl) and 2.0 μl of sample DNA. The LAMP reaction was performed at 65°C for 60 min and then terminated by heating the mixture at 80°C for 5 min. The turbidity in the reaction tube used in the LAMP method is proportional to the amount of amplified DNA. Therefore, the LAMP products were detected by monitoring the turbidity with a real-time turbidimeter (LA-320C, Eiken Chemical Co., Ltd.).

We examined 172 strains, all of which except for a type strain of *S. zooepidemicus* (ATCC 43079^T) were isolated from diseased horses between 1980 and 2013. The 172 strains comprised 51 strains of *S. zooepidemicus*, 50 strains of *S. equi*, 23 strains of *Streptococcus dysgalactiae* subsp. *equisimilis*, 18 strains of other *Streptococcus* species including *Streptococcus pneumonia*, which is generally human pathogenic bacterium, and 30 strains belonging to other genera frequently isolated from horses (Table 2). Each bacterial DNA was extracted by using InstaGene Matrix (Bio-Rad Laboratories, Tokyo, Japan) in accordance with the manufacturer's instructions.

Amplification of the LAMP products originating from the 51 strains of *S. zooepidemicus* was confirmed. By contrast, no amplification of the LAMP products originating from the *S. equi* strains or from other streptococci or other genera was confirmed.

The process of DNA extraction from clinical samples is necessary for various genetic tests, including LAMP. However, veterinary practitioners are unable to use most DNA extraction methods, because they require complicated processes that are time-consuming and labor-intensive. Here, we also compared the results obtained when LAMP was used with each of 3 DNA extraction methods that veterinary practitioners could apply easily to clinical samples.

To measure the detection limits of the LAMP method in combination with each of 3 different DNA extraction methods, 10-fold serial dilutions of a suspension of *S. zooepidemicus* were prepared, and the colony forming units (CFUs) in the suspension were counted. Bacterial DNA in the suspensions was then extracted by using one of 3 DNA extraction methods, namely a Loopamp PURE DNA Extraction Kit (Eiken Chemical Co., Ltd.), InstaGene Matrix or a boiling method. These DNA extraction methods were

Table 3. Comparison of efficacies of the 3 methods of extracting DNA from clinical and spiked samples

Sample	*S. zooepidemicus* concentration (CFU/m*l*)	Number	Numbers (%) of LAMP-positive samples		
			Loopamp PURE DNA Extraction kit	InstaGene matrix	Boiling method
Clinical sample	<20 [b)]	34	1 (2.9)	1 (2.9)	0
	20 to 1×10^4	6	0	1 (16.6)	1 (16.6)
	$>1 \times 10^4$	14	13 (92.9)	11 (78.6)	7 (50)
Spiked sample [a)]	1×10^6	34	34 (100)	31 (91.2)	27 (79.4)

a) Type strain of *S. zooepidemicus* (ATCC 43079^T) was added to clinical samples with *S. zooepidemicus* concentrations less than 20 CFU/m*l*. b) Detection limit of our bacterial culture was 20 CFU/m*l*.

selected, because they could be performed with only a few steps and within 30 min. DNA was extracted with the Loopamp PURE DNA Extraction Kit or InstaGene Matrix in accordance with the manufacturer's instructions. With the boiling method, samples were boiled at 100°C for 10 min and were centrifuged at 13,000 g for 3 min. The aliquots obtained from the supernatant were then subjected to LAMP analysis. Sensitivity tests were performed 3 times for each DNA extraction method; we regarded the lowest bacterial concentrations that yielded positive results at least twice as the detection limits (CFU/m*l*).

The detection limits of the LAMP method in combination with the Loopamp PURE DNA Extraction Kit, InstaGene Matrix and boiling method for pure culture were 1×10^5, 1×10^3 and 1×10^4 CFU/m*l*, respectively. These detection limits (CFU/m*l*) of the Loopamp PURE DNA Extraction Kit, InstaGene Matrix and boiling method are equal to 200 CFU/reaction, 2 CFU/reaction and 20 CFU/reaction, respectively.

The clinical efficacy of a genetic test can sometimes differ from the results of sensitivity testing in *in vitro* experiments using pure bacterial culture [15, 16], because each DNA extraction method has a different performance in terms of DNA purification or the yield of DNA extracted from clinical specimens. Therefore, we used clinical samples and spiked samples to assess the clinical efficacies of the 3 DNA extraction methods. Of the 54 clinical samples obtained from the respiratory tracts of Thoroughbred horses, one was obtained in 2010, 25 in 2012 and 28 in 2013. The 54 clinical samples consisted of 33 bronchoalveolar lavage (BAL) fluids, 12 tracheal washes, 8 pleural effusions and 1 guttural pouch lavage specimen. The clinical samples were suspended in equal amount of Cary-Blair medium and were transported to our laboratory. One hundred μl of the mixtures were incubated aerobically on 5% horse blood agar at 37°C for 24 hr. The remaining samples were stored at −20°C until DNA isolation. In a previous study, 69 of 148 healthy Thoroughbred horses had bacteria in their tracheas at concentrations of 1 to 10^4 CFU/m*l*; the concentration of the bacteria was greater than 1×10^4 CFU/m*l* in only seven of these horses [6]. In another previous study, the average concentration of bacteria in tracheal washes from Standardbred horses with no clinical signs was 1.49×10^4 CFU/m*l* [12]. Moreover, the presence of bacteria at concentrations greater than 1×10^4 CFU/m*l* in human BAL samples is associated with clinical disease [3]. We therefore considered a concentration of greater than 1×10^4 CFU/m*l* in clinical samples to be of clinical importance. Thus, we divided the clinical samples into 3 groups by using 2 thresholds: 20 CFU/m*l* (the detection limit of our bacterial culture) and 1×10^4 CFU/m*l*. Thirty-four of 54 clinical samples yielded *S. zooepidemicus* at less than 20 CFU/m*l*. The concentration of *S. zooepidemicus* was between 20 CFU/m*l* and 1×10^4 CFU/m*l* in 6 of 54 samples and greater than 1×10^4 CFU/m*l* in 14 of 54 samples. To prepare the spiked samples, 34 clinical samples with *S. zooepidemicus* concentrations of less than 20 CFU/m*l* were spiked at one-tenth their volumes with a suspension in which the concentration of *S. zooepidemicus* (ATCC 43079^T) was 1×10^7 CFU/m*l*. The final concentration of *S. zooepidemicus* in each spiked sample was at least 1×10^6 CFU/m*l*.

Thirteen of 14 clinical samples with *S. zooepidemicus* concentrations greater than 1×10^4 CFU/m*l* were positive by the LAMP method plus the Loopamp PURE DNA Extraction Kit; 11 of these 14 clinical samples were positive with LAMP plus InstaGene Matrix, and 7 were positive with LAMP plus boiling (Table 3). Thirty-four of 34 spiked samples were positive with LAMP plus the Loopamp PURE DNA Extraction Kit; 31 were positive with LAMP plus InstaGene Matrix and 27 with LAMP plus boiling. Among the 40 clinical samples in which *S. zooepidemicus* concentrations were either less than 20 CFU/m*l* or 20 to 1×10^4 CFU/m*l*, one clinical sample was positive with LAMP plus the Loopamp PURE DNA Extraction Kit, 2 with LAMP plus InstaGene Matrix and one with LAMP plus boiling.

Although the sensitivity of the LAMP method plus the Loopamp PURE DNA Extraction Kit was 10 to 100 times lower than those with InstaGene Matrix or boiling, the combination of LAMP plus the Loopamp PURE DNA Extraction Kit could detect *S. zooepidemicus* in both clinical and spiked samples more efficiently than the other methods. Because the bacterial concentrations in the spiked samples were sufficient to give positive results by LAMP in pure culture, the LAMP method would theoretically be expected to give positive results in all spiked samples. However, not all spiked samples were positive by LAMP plus InstaGene Matrix or the boiling method; 100% detection was obtained only with the Loopamp PURE DNA Extraction Kit. Many inhibitors are present in specimens from the respiratory tract, and they can cause false-negative results in PCR [11, 26]. These inhibitory substances either bind to polymerase or interact with the sample's DNA or polymerase or both, dur-

ing primer extension [20, 26]. The inhibitors remaining after DNA extraction using InstaGene Matrix or boiling might inhibit the LAMP reaction; using the Loopamp PURE DNA Extraction Kit would be more likely to reduce the effect of these inhibitors than would the other extraction methods. Our results suggest that the InstaGene Matrix and boiling methods are unsuitable for use on clinical samples because they can yield false negatives; the LAMP method plus the Loopamp PURE DNA Extraction Kit is the most appropriate for use on clinical samples. LAMP plus the Loopamp PURE DNA Extraction Kit gave a positive result in one clinical sample in which the *S. zooepidemicus* concentration was less than 20 CFU/m*l*, despite the fact that the detection limit of this combination in pure culture was 1×10^5 CFU/m*l*. This inconsistency may have been caused by the presence of dead *S. zooepidemicus* in the sample, because the samples were collected after the administration of antimicrobials.

In conclusion, we developed a LAMP method for detecting *S. zooepidemicus*. This method can yield results within 1 hr, and unlike previous PCR-based methods, it can be applied to clinical samples without the need for special equipment. The LAMP method in combination with the Loopamp PURE DNA Extraction Kit is efficient for detecting *S. zooepidemicus* in clinical and spiked samples containing clinically significant concentrations of *S. zooepidemicus*, and it takes a total of only about 90 min from receiving the specimens to obtaining the LAMP results. Therefore, veterinary practitioners can diagnose *S. zooepidemicus* pneumonia in horses sooner with the LAMP method—even in laboratories with only basic equipment—and can select proper treatment in the early phase of infection.

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