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Development and Application of Loop-Mediated Isothermal Amplification Methods Targeting the *seM* Gene for Detection of *Streptococcus equi* subsp. *equi*

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ABSTRACT. Loop-mediated isothermal amplification (LAMP) constitutes a potentially valuable diagnostic tool for rapid diagnosis of contagious diseases. In this study, we developed a novel LAMP method (*seM*-LAMP) to detect the *seM* gene of *Streptococcus equi* subsp. *equi* (*S. equi*), the causative agent of strangles in equids. The *seM*-LAMP successfully amplified the target sequence of the *seM* gene at 63°C within 60 min. The sensitivity of the *seM*-LAMP was slightly lower than the 2nd reaction of the *seM* semi-nested PCR. To evaluate the species specificity of the *seM*-LAMP, we tested 100 *S. equi* and 189 non-*S. equi* strains. Significant amplification of the DNA originating from *S. equi* was observed within 60 min incubation, but no amplification of non-*S. equi* DNA occurred. The results were identical to those of *seM* semi-nested PCR. To investigate the clinical usefulness of the methods, the *seM*-LAMP and the *seM* semi-nested PCR were used to screen 590 nasal swabs obtained during an outbreak of strangles. Both methods showed that 79 and 511 swabs were *S. equi* positive and negative, respectively, and the results were identical to those of the culture examination. These results indicate that the *seM*-LAMP is potentially useful for the reliable routine diagnosis of *Streptococcus equi* subsp. *equi* infections.
KEY WORDS: equine, loop-mediated isothermal amplification, *seM* gene, strangles, *Streptococcus equi.*

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Strangles is an infectious disease of equids caused by *Streptococcus equi* subsp. *equi* (*S. equi*) and is a commonly diagnosed and important infectious disease of horses worldwide [2, 6–8, 13–16]. It is characterized by a mucopurulent nasal discharge and acute swelling with subsequent abscess formation in the submandibular and retropharyngeal lymph nodes [5, 13–15]. *S. equi* infects the horse's cranial lymph glands and is highly communicable to other horses. Outbreaks of strangles have been reported in many countries and can cause scheduled horse racing meets to be cancelled [4, 6–8, 11]. *S. equi* produce an M-like protein, SeM, and the *seM* gene has been proposed as a target gene to distinguish *S. equi* from different strains of streptococci including *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) [15, 16].

An accurate diagnostic test for strangles would be of great potential benefit for clinical purposes because early recognition and monitoring would be possible from the results. However, the diagnostic procedure conventionally used as a culture examination for *S. equi* requires significant time before the result is obtained. The alternative PCR method that detects the *S. equi* gene to confirm the diagnosis genetically can be applied clinically [1, 4, 11]; however, significant time is required to conduct the PCR method, although the disease can be diagnosed more promptly with PCR than by culture. The development of a rapid diagnostic method is highly desirable to alleviate these delays.

The loop-mediated isothermal amplification (LAMP) method was developed as a nucleic acid amplification method to replace the PCR method, and the LAMP method has been clinically applied as a rapid diagnostic method for various pathogens in recent years [9, 10, 12, 17, 18]. LAMP amplifies a nucleic acid with high specificity, efficiency, and speed under isothermal conditions. Therefore, it constitutes a potentially valuable diagnostic tool for rapid diagnosis of contagious diseases.

In this study, we developed a novel LAMP method (*seM*-LAMP) to detect the *seM* gene of *S. equi* and demonstrated usefulness of the method as a rapid diagnostic tool for strangles.

MATERIALS AND METHODS

Bacterial strains and preparation of DNA: The bacteria used in this study were 100 strains of *S. equi*, 100 strains of *S. zooepidemicus* and 89 strains of various other bacteria, all of which had been isolated from various clinical samples of horses between 1980 and 2010 and stored at –80°C (Table 1). The bacteria were suspended in the lysis buffer of a nucleic acid extraction kit (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, U.S.A.). Bacterial DNA was then extracted according to the manufacturer's instructions and stored at –80°C for use in this study as a template for LAMP or PCR amplification.

Primer design for seM-LAMP: The *seM*-LAMP primers were designed based on the published sequences of the *seM* gene (GenBank accession numbers, U73162, AF012927, AJ249868) by using the PrimerExplorer V4 software

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Table 1. Bacterial strains used in this study

Bacteria	Number of strains
Streptococcus equi subsp. *equi*	100
Streptococcus equi subsp. *zooepidemicus*	100
Streptococcus dysgalactiae subsp. *equisimilis*	21
Streptococcus acidominimus	7
Streptococcus mitis	3
Streptococcus pyogenes	3
Streptococcus bovis	2
Streptococcus equinus	2
Streptococcus constellatus subsp. *constellatus*	1
Streptococcus epidermidis	1
Other Streptococci	4
Escherichia coli	10
Staphylococcus aureus	10
Klebsiella pneumoniae	10
Pseudomonas aeruginosa	10
Enterobacter aerogenes	1
Salmonella Abortusequi	1
Actinobacillus equuli	1
Bordetella bronchiseptica	1
Rhodococcus equi	1

(Fujitsu Limited, Tokyo, Japan). The consensus sequence among the *S. equi* strains was elucidated, and *seM*-LAMP primers were designed using the alignment analysis. Five primers comprising two outer primers (F3 and B3), two inner primers (FIP and BIP) and one loop primer (Loop F) were designed (Table 2).

LAMP reaction: The reaction mixture was prepared using a DNA amplification kit (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 with 12.5 μl of 2X reaction mix buffer, 0.2 μM each of 2 outer primers (F3 and B3), 1.6 μM each of 2 inner primers (FIP and BIP), 0.8 μM loop primer (Loop F), 1.0 μl of *Bst* DNA polymerase (8 units/μl) and 2.0 μl of sample DNA. The LAMP reaction was performed at 63°C for 60 min and then terminated by heating the mixture at 80°C for 5 min.

Analysis of LAMP products: The LAMP reaction causes turbidity in the reaction tube proportional to the amount of amplified DNA. Therefore, *seM*-LAMP products were detected by monitoring the turbidity using a real-time turbidimeter (LA-320C, Eiken Chemical Co., Ltd.). To confirm the sequence of the amplified *seM*-LAMP products, some of the amplified products were sequenced using a sequencing kit (BigDye Terminator V3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, U.S.A.) and a DNA Sequencer (AJ31 PRISM 377 DNA sequencer, Applied Biosystems) in accordance with the manufacturers' instructions. The outer primers (F3, B3) were used to sequence the target region (between F3 and B3) (Table 3). To clarify the detection limit of the *seM*-LAMP assay for *S. equi*, serial 10-fold dilutions of a mid-log phase *S. equi* strain CF32 culture (10^8 colony forming units (CFU)/m*l*) grown in Todd-Hewitt broth (BD Japan, Tokyo, Japan) were prepared in 10 mM phosphate buffered saline and quantified using the standard plating method. DNA templates were prepared from each dilution by the nucleic acid extraction kit described above, and aliquots (2 μl) were subjected to both LAMP and PCR amplifications. Sensitivity tests were repeated three times, and the mean limits of detection (CFU/reaction) were reported. For further confirmation, the amplified products in the detection limit test were also detected using electrophoresis in 2.0% agarose gels, followed by ethidium bromide staining

Semi-nested-PCR: The semi-nested PCR targeting the *seM* gene (*seM* semi-nested PCR) was performed according to a previous report [1] with some modifications. In brief, initial PCR was performed in a 25 μl volume containing 2.0 μl of template DNA solution, 0.4 mM each of SEf and SEr1 PCR primer (Table 2), 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphates and 0.6 U of DNA polymerase (z-Taq, Takara Bio Inc., Shiga, Japan). Samples were amplified by a one denaturation step at 95°C for 3 min followed by 30 amplification cycles consisting of denaturation at 98°C for 1 sec, annealing at 68°C for 5 sec, and elongation at 70°C for 3 min in a thermal cycler. The 289 bp region was further amplified from the initial PCR product with a second primer set, SEf-SEr5 (Table 2), under the same reaction conditions as described above. Amplicons were analyzed using 2.0% agarose gel electrophoresis.

Field samples and preparation of DNA: To investigate

Table 2. Primers of *seM*-LAMP and *seM*-semi-nested PCR for detection of *S. equi*

	Primer	Sequence (5'→3')	Genome position[a)]	Reference
seM-LAMP	F3	CAGCCCAAAAAGTTCGAAAT	538–557	This study
	B3	GCTTTTCAATTTCATCTACAAGT	725–747	
	FIP[b)]	GCCCTTGCTGAATCAAGACCTCTTCTAAAAGGCGCCTCTG	602–622(F1c) - 558 –576(F2)	
	BIP[c)]	TTGATGCACCTTTCATCGATGTTGAAGCCAAACTTAATTGTCT	654–676(B1c) – 702–21(B2)	
	Loop F	CTCAATAATGCCTGTAAATCCCCAA	577–601	
seM semi-nested PCR	SEf	AAAGATGAGCGTCAAGCTCTTACC	888–911	Anzai *et al.*[1)]
	SEr1	TGCTTTTTGTTTTTCAAGCTCTGCTA	1468–1493	
	SEr5	CAGCTTCTGCTGTTTTAGCTGCCA	1153–1176	

a) Positions of *seM*-LAMP primers from the start codon of the *S. equi seM* gene (Accession number: U73162).
b) FIP primer consists of F1c and F2 regions.
c) BIP primer consists of B1c and B2 regions.

the clinical usefulness of the methods, the *seM*-LAMP and the *seM* semi-nested PCR were used to screen 590 nasal swabs obtained during an outbreak of strangles [6]. Each nasal swab was suspended in 500 μl normal saline solution, and 50 μl of the suspension solution was used for bacterial extermination. *S. equi* was isolated by inoculating Columbia CNA agar (BD Japan) containing 5% horse blood with 50 μl of the suspension, and *S. equi* was identified using API Strep 20 (SYSMEX bioMerieux, Tokyo, Japan). Fifty μl of the suspension was applied for DNA extraction as described above.

RESULTS

Reactivity of the seM-LAMP: The results of real-time turbidity measurements in the LAMP reaction solutions containing 0.01 to 10^4 CFU/reaction of *S. equi* strain CF32 are shown in Fig. 1. Increasing the quantity of initial template DNA shortened the time to reach the threshold time. The *seM*-LAMP was able to detect the *seM* gene of *S. equi* within 43 min if 0.1 CFU/reaction of *S. equi* was present and within 23 min if 10^4 CFU/reaction or more of template DNA was present. The detection limits for the *seM*-LAMP and *seM* semi-nested PCR were 0.1 and 0.01 CFU/reaction, respectively. No amplification was apparent when the sample tube did not contain target DNA. A plot of the amplification time required to exceed a turbidity level of 0.1 (threshold time) versus the log CFU/reaction showed a linear relationship, with a high coefficient of determination (r^2=0.988) (Fig. 1). The products of the *seM*-LAMP were evident upon agarose gel electrophoresis as a ladder-like pattern on the gel, which is characteristic of the LAMP (Fig. 1). The sequences of the amplified products matched the expected nucleotide sequences of the *seM* gene (bases 538 to 747 in the original sequence) perfectly.

Specificity of the seM-LAMP: To evaluate the species and subspecies specificity of the *seM*-LAMP, we tested 100 *S. equi* and 189 non-*S. equi* isolates (Table 1). Significant amplification of the DNA originating from *S. equi* was observed within 60 min of incubation. By contrast, none of the DNA originating from other strains was amplified, even after 60 min of incubation. The results were identical to the results with *seM* semi-nested PCR.

Application of the seM-LAMP for clinical use: To investigate the clinical usefulness of the methods, the *seM*-LAMP and the *seM* semi-nested PCR were used to screen 590 nasal swabs obtained during an outbreak of strangles. By culture examination, *S. equi* was isolated from 79 of the 590 nasal swabs. The results of the *seM*-LAMP and *seM* semi-nested PCR were identical to those of the culture examination; that is, the 79 *S. equi*-positive swabs showed a positive reaction, but the rest of the swabs did not. By the *seM*-LAMP, the positive reactions in the 79 swabs were observed within 33 min.

DISCUSSION

Strangles is an extremely important disease in horses because of its extreme contagiousness [13, 14]. Therefore, rapid diagnosis is essential for horses in which the appearance of strangles is questioned. As a result, we focused on developing the LAMP method as a diagnostic technique that could be faster and easier to perform than the PCR method, and we evaluated the technique in this study.

The LAMP method had slightly lower sensitivity than the 2nd reaction of the *seM* semi-nested PCR when the *seM*-LAMP and the *seM* semi-nested PCR method were compared. However, this is unlikely a problem for clinical application because the LAMP method was able to detect the *seM* gene of *S. equi* with 0.1 CFU/reaction. Moreover, the *seM*-LAMP offers a great advantage in requiring only about 1.5 hours to obtain results, whereas the *seM* semi-nested PCR requires about four hours from DNA extraction to obtaining the results.

It is a concern that nonspecific positive reactions from assays can be elicited because of the numbers of various species of bacteria that exist in a healthy horse's nasal cavity and tonsils [3]. However, neither *S. zooepidemicus* nor any of the other resident bacteria in the nasal cavity showed positive reactions by the *seM*-LAMP. This finding suggests that the specificity of the *seM*-LAMP is extremely high.

The *seM*-LAMP was applied to samples obtained during an outbreak of strangles [6]. The results of the *seM*-LAMP were identical to those of the culture examination and *seM* semi-nested PCR of *S. equi*. This finding suggests that in future strangles outbreaks, use of the *seM*-LAMP developed in this study will benefit the response by reducing detection time. Moreover, the *seM*-LAMP can produce similar results using a kit for visual analysis of turbidity and an incubator to maintain constant temperature even if the real-time turbidimeter used in this study is not used [9]. Based on this, the potential for applying the *seM*-LMAP in clinical situations seems promising.

In conclusion, we have developed an *seM*-LAMP-based *S. equi* DNA amplification method, tested it and determined that its reliability in species discrimination is very high. The *seM*-LAMP is potentially useful for the reliable routine diagnosis of *S. equi* infections.

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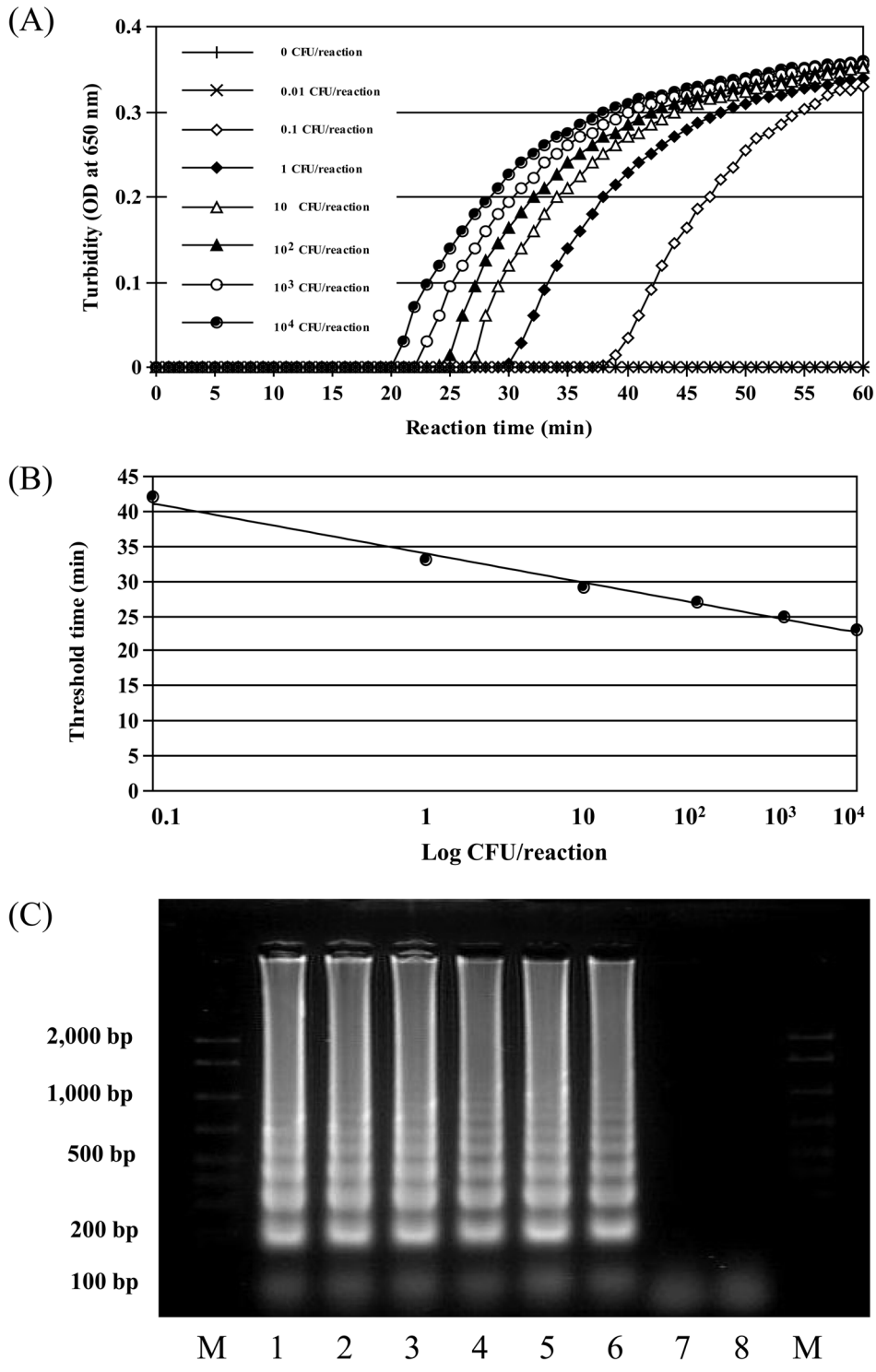

Fig. 1. Real-time sensitivity and detection limit of *seM*-LAMP. (A) Real-time sensitivity of *seM*-LAMP monitored by the measurement of turbidity (optimal density (OD) at 650 nm). A turbidity of >0.1 was considered to be positive for *seM*-LAMP. The detection limit was 0.1 CFU/reaction. (B) The relation between the threshold time (Tt) of each sample and the log CFU/reaction. (C) Sensitivities of electrophoretic analysis of *seM*-LAMP amplified products. Lane M: ladder. Lane 1: 10^4 CFU/reaction *S. equi* CF32. Lane 2: 10^3 CFU/reaction. Lane 3: 10^2 CFU/reaction. Lane 4: 10 CFU/reaction. Lane 5: 1 CFU/reaction. Lane 6: 0.1 CFU/reaction. Lane 7: 0.01 CFU/reaction. Lane 8: no template.

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