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Development of loop-mediated isothermal amplification methods for detecting *Taylorella equigenitalis* and *Taylorella asinigenitalis*

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Taylorella equigenitalis is a causative bacterium of contagious equine metritis (CEM), and Taylorella asinigenitalis is species belonging to genus Taylorella. The authors developed two loop-mediated isothermal amplification (LAMP) methods, Te-LAMP and Ta-LAMP, for detecting T. equigenitalis and T. asinigenitalis, respectively. Using experimentally spiked samples, Te-LAMP was as sensitive as a published semi-nested PCR method, and Ta-LAMP was more sensitive than conventional PCR. Multiplex LAMP worked well without nonspecific reactions, and the analytical sensitivities of multiplex LAMP in the spiked samples were almost equivalent to those of Te-LAMP and Ta-LAMP. Therefore, the LAMP methods are considered useful tools to detect T. equigenitalis and/or T. asinigenitalis, and preventive measures will be rapidly implemented if the occurrence of CEM is confirmed by the LAMP methods.

Key words: *contagious equine metritis, loop-mediated isothermal amplification, Taylorella asinigenitalis, Taylorella equigenitalis*

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Taylorella equigenitalis is a Gram-negative coccobacillus classified in the *Alcaligenaceae* family [12]. It is the causative agent of contagious equine metritis (CEM), a sexually transmitted infection of horses. CEM was first reported in 1977 [3, 15], and it has been detected in many countries and various horse breeds [10, 11, 13, 14]. The characterizations of mares infected by CEM are abundant mucopurulent vaginal discharge and a variable degree of vaginitis, endometritis, and cervicitis, usually resulting in temporary infertility [14]. No clinical signs are observed in stallions, and asymptomatic carrier mares have been reported [4].

Atypical *T. equigenitalis* was isolated in 1997–1998 from two male donkeys and a mare with no clinical signs [6]. This newly identified bacterium was classified following taxonomic studies as a new species named *Taylorella asinigenitalis* [5]. *T. asinigenitalis* has not been associated with naturally occurring disease in horses [5, 7], and only the detection of *T. equigenitalis* in a horse has so far led to the formal declaration of occurrence of CEM.

A few polymerase chain reaction (PCR)-based methods for detecting *T. equigenitalis* have been described [1, 2]. However, it is difficult to use PCR-based methods in less well-equipped laboratories, because special equipment such as a thermal cycler is needed. The loop-mediated isothermal amplification (LAMP) method was developed as a new nucleic acid amplification method [9]. LAMP amplifies nucleic acids with high speed, specificity, and efficiency, and it can be performed under isothermal conditions with no special equipment. The LAMP method is potentially valuable as a diagnostic tool for rapid diagnosis of contagious diseases in less well-equipped laboratories, and the authors considered that LAMP assays would be useful tools for detecting *T. equigenitalis* and *T. asinigenitalis* in less well-equipped laboratories.

In this study, novel LAMP methods were developed, namely Te-LAMP and Ta-LAMP, targeting the 23S rRNA genes of *T. equigenitalis* and *T. asinigenitalis*, respectively. The authors evaluated the analytical sensitivity of these

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Table 1. Primer sets used in this study

Method	Primer	Sequence (5′→3′)
Te-LAMP	F3	AAAGTAGTGGCGAGCGAAAT
	B3	ACTATCGGTCGATCACGAGT
	FIP[a)]	ACGGTCGCACTTCCCAATGCTGGAGTAGCCGCAACGAG
	BIP[b)]	GCGTTGCAACAAGTAGGGCGGAGGATGGTCCCCCCATA
	Loop B	GGACACGTGTAATCCTGTTCG
Ta-LAMP	F3	CTGAATACATAGGGTGAGGAGG
	B3	TTGCAACGCCTAGTACCATA
	FIP[a)]	CGCCACTACTTTCAGAATCTCGGTCGAACCGAGTGAACTGAAACA
	BIP[b)]	AACGAAATTGGAGCAGCCGCAACTATCACCCTCTACGGTCTT
	Loop B	AATTGTAGTAGTCGAACTTACTGGG

a) The FIP primer consists of F1c and F2 regions, b) The BIP primer consists of B1c and B2 regions.

Table 2. Bacterial strains used in this study

Bacteria	Number of strains
Taylorella equigenitalis (NCTC 11184^T and 66 clinical strains[a)])	67
Taylorella asinigenitalis (ATCC 700933^T and UK -1)	2
Achromobacter xylosoxidans[b)] (JCM 9659^T)	1
Alcaligenes faecalis[b)] (JCM 20522^T)	1
Azohydromonas lata[b)] (JCM 20675^T)	1
Bordetella pertussis[b)] (ATCC 9797^T)	1
Pusillimonas noertemannii[b)] (DSM 10065^T)	1
Bordetella bronchiseptica[b)]	3
Streptococcus equi subsp. *zooepidemicus*[c)]	3
Staphylococcus aureus[c)]	3
Klebsiella pneumoniae[c)]	3
Escherichia coli[c)]	3
Pseudomonas aeruginosa[c)]	3

a) Sixty-six clinical strains were isolated in Japan between 1980 and 2000, b) These strains and genus *Taylorella* belong to the same family (*Alcaligenaceae*), c) These species are frequently isolated from specimens of horses.

LAMP methods, compared with previously described PCR-based methods. In addition, a combination of Te-LAMP primers and Ta-LAMP primers, as a multiplex method, was attempted to detect either the genes of *T. equigenitalis* or *T. asinigenitalis*, or both, in the same reaction tube.

Both Te-LAMP and Ta-LAMP primers were designed on the basis of the published sequences of the 23S rRNA genes by using the PrimerExplorer V4 software (Fujitsu Limited, Tokyo, Japan) (Table 1). The GenBank accession numbers of the 23S rRNA genes used for Te-LAMP were AB277176, AB277177, and AB277178. The GenBank accession numbers of the 23S rRNA genes used for Ta-LAMP were AB277175, AB277170, AB277172, and AB277173.

The reaction mixture was prepared by using a DNA amplification kit (Loopamp DNA Amplification Kit, Eiken Chemical Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's instructions. The Te-LAMP and Ta-LAMP reactions were performed for 60 min at 64°C and 63°C, respectively. Multiplex LAMP using both Te-LAMP and Ta-LAMP primers was performed at 64°C for 60 min. Multiplex LAMP detects both *T. equigenitalis* and *T. asinigenitalis* and cannot distinguish between them. The LAMP products were detected by monitoring the turbidity with a real-time turbidimeter (LA-320C, Eiken Chemical Co., Ltd.).

Ninety-two strains were used for confirming the specificity in the study (Table 2). Bacterial DNA was extracted by using a commercial DNA extraction kit (InstaGene Matrix, Bio-Rad Laboratories, Tokyo, Japan) in accordance with the manufacturer's instructions. The DNA concentrations were measured by a fluorometer (Qubit, Invitrogen Japan KK, Tokyo, Japan), and the gene copy numbers of each DNA extract were estimated by its DNA concentrations [16].

To measure the detection limits of the Te-LAMP and Ta-LAMP, 10-fold serial dilutions of each purified DNA extract were prepared in 0.1 × TE buffer; the original concentrations of purified DNA extract from *T. equigenitalis* (NCTC 11184^T) and *T. asinigenitalis* (ATCC

Table 3. Comparison among LAMP and PCR-based methods for detecting *T. equigenitalis* DNA in spiked samples

Test	Positive numbers in spiked sample[b)]			Positive numbers in non-spiked samples
	1.24×10^2 copies/μl[c)]	1.24×10 copies/μl	1.24 copies/μl	
Te-LAMP	22 / 22	21 / 22	12 / 22	0/192
Multiplex LAMP[a)]	22 / 22	22 / 22	15 / 22†	0/192
PCR	22 / 22	22 / 22	7 / 22	0/192
Semi-nested PCR	21 / 22	20 / 22	17 / 22†	0/192

a) Multiplex LAMP consists of Te-LAMP primers and Ta-LAMP primers in a single reaction tube, b) Fisher's exact test was conducted for statistical analysis of comparisons between the assays for each concentration of spiked samples. A *P*-value ≤ 0.05 was considered to indicate a significant difference in this study, c) The concentration indicates the gene copy number of *T. equigenitalis* (NCTC 11184^T), †) The number of positive samples was significantly large compared with that for PCR ($P<0.05$, Fisher's exact test).

Table 4. Comparison among LAMP and PCR-based methods for detecting *T. asinigenitalis* DNA in spiked samples

Test	Positive numbers in spiked sample[b)]			Positive numbers in non-spiked samples
	2.36×10^2 copies/μl[c)]	2.36×10 copies/μl	2.36 copies/μl	
Ta-LAMP	22 / 22*	22 / 22*	5 / 22*	0/192
Multiplex LAMP[a)]	21 / 22*	20 / 22*	9 / 22*	0/192
PCR	12 / 22	2 / 22	0 / 22	0/192

a) Multiplex LAMP consists of Te-LAMP primers and Ta-LAMP primers in a single reaction tube, b) Fisher's exact test was conducted for statistical analysis of comparisons between the assays for each concentration of spiked samples. A *P*-value ≤ 0.05 was considered to indicate a significant difference in this study, c) The concentration indicates the gene copy number of *T. asinigenitalis* (ATCC 700933^T), *) The number of positive samples was significantly large compared with that for PCR ($P<0.05$, Fisher's exact test).

700933^T) were 1.24×10^6 copies/μl and 2.36×10^6 copies/μl, respectively. The aliquots were then subjected to the LAMP assays. Sensitivity tests were performed three times for each LAMP assay; the lowest bacterial concentrations that yielded positive results at least twice were regarded as the detection limits (copies/reaction).

Amplification of the LAMP products originating from the 67 *T. equigenitalis* strains and two *T. asinigenitalis* strains by Te-LAMP and Ta-LAMP, respectively, was confirmed. By contrast, no amplification of LAMP products originating from the other strains or from non-spiked clinical samples, as described below, was confirmed by Te-LAMP or Ta-LAMP (Tables 3 and 4).

The *in vitro* sensitivity of Te-LAMP for detecting *T. equigenitalis* was 24.8 copies/reaction, and that of Ta-LAMP for detecting *T. asinigenitalis* was 47.2 copies/reaction.

Two PCR-based methods—namely PCR [2] and semi-nested PCR [1], were used to compare the analytical sensitivity for experimentally spiked samples. The PCR assay detects both *T. equigenitalis* and *T. asinigenitalis* and cannot distinguish between them [8], and the semi-nested PCR assay detects only *T. equigenitalis* genes (unpublished data). The semi-nested PCR assay was performed according to a previous report [1]. The PCR assay was conducted according to the published method [2] with slight modifications. In brief, the PCR assay was performed in 50 μl of reaction mixture containing 2 μl of sample DNA, 25 μl of 2 × premix (EmeraldAmp PCR Master Mix, Takara Bio Inc., Kyoto, Japan), and oligonucleotide primers (0.1 μM) and was conducted as follows: initial denaturation (94°C, 5 min); 35 cycles of denaturation (94°C, 30 sec), annealing (55°C, 15 sec), and extension (72°C, 1 min); and then a final elongation at 72°C for 7 min. The DNA volumes used for LAMP, PCR, and semi-nested PCR assays, as described above, were 2 μl, 2 μl, and 5 μl, respectively.

To compare the analytical sensitivities of the Te-LAMP, Ta-LAMP, multiplex LAMP, PCR, and semi-nested PCR assays, 324 genital swabs of the clitoral fossa and clitoral sinuses from Thoroughbred mares were used. Each genital swab was suspended in 100 μl distilled water and then boiled at 100°C for 10 min. Sixty-six samples were randomly allocated to three groups (i.e., each group had 22 samples), and the samples in each group were spiked with 10-fold serial dilutions of DNA extracts of *T. equigenitalis* (NCTC 11184^T). The DNA concentrations of each group were 1.24, 1.24×10, and 1.24×10^2 copies/μl, respectively. Another 66 samples were spiked with 10-fold serial dilutions of DNA extracts of *T. asinigenitalis* (ATCC 700933^T) in the same manner as *T. equigenitalis*. The DNA concentrations of each group were 2.36, 2.36×10, and 2.36×10^2 copies/μl, respectively. The other 192 swabs were used as non-spiked clinical samples. All 324 genital swabs were preliminarily

confirmed as *T. equigenitalis* negative by the semi-nested PCR, the official analytical method used for CEM in Japan.

The results of samples spiked with *T. equigenitalis* indicated that the positive numbers obtained with multiplex LAMP and semi-nested PCR were significantly higher than that of the PCR with the concentration of 1.24 copies/*µl* (Table 3). Te-LAMP showed no significant difference compared with any other tests, indicating that Te-LAMP was as sensitive as semi-nested PCR, the official analytical method in Japan, when used on spiked samples. For *T. asinigenitalis*, Ta-LAMP and multiplex LAMP showed significantly higher positive numbers than PCR (Table 4).

Here, the authors developed two specific LAMP assays, Te-LAMP and Ta-LAMP, for detecting *T. equigenitalis* and *T. asinigenitalis*, respectively. The PCR assay [2] developed for detecting *T. equigenitalis* could also detect *T. asinigenitalis* [8], although semi-nested PCR [1] could specifically detect *T. equigenitalis* but could not detect *T. asinigenitalis* ATCC 700933^T. The Te-LAMP could specifically detect *T. equigenitalis* as well as the semi-nested PCR, and the reaction time for Te-LAMP (60 min) was shorter than that for the semi-nested PCR (a few hours). To evaluate the analytical sensitivity of these gene amplification methods, experimentally spiked samples were used in this study. The experimentally spiked samples were considered suitable for the purpose of comparing the analytical sensitivities because the spiked samples and clinical samples contained many inhibitors of gene amplification. The results with the spiked samples indicated that Te-LAMP was as sensitive as both the semi-nested PCR and PCR in spiked samples. LAMP requires no special equipment, and the results can be judged visually by using a fluorescent detection reagent. The authors therefore concluded that Te-LAMP could be easily introduced to less well-equipped laboratories as a highly sensitive method of detecting *T. equigenitalis* in clinical samples, thus allowing preventive measures for CEM to be implemented rapidly if a positive result is obtained by Te-LAMP. In the case of *T. asinigenitalis*, Ta-LAMP was more specific than the PCR and more sensitive in spiked samples. Therefore, Ta-LAMP was considered the most useful method for detecting *T. asinigenitalis* in spiked samples.

The authors also found that the sensitivities of the multiplex LAMP for spiked samples were close to those of Te-LAMP and Ta-LAMP. Currently, detection of *T. asinigenitalis* in a horse does not lead to declaration of an outbreak of CEM, although mares experimentally infected with *T. asinigenitalis* can develop clinical signs of metritis and cervicitis [6]. It is possible that, in the future, a declaration of occurrence of CEM will be based on detection of either *T. equigenitalis* or *T. asinigenitalis*. Therefore, multiplex LAMP could become a useful tool for easier screening for both *T. equigenitalis* and *T. asinigenitalis*.

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