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A New Loop-Mediated Isothermal Amplification Method for Rapid, Simple, and Sensitive Detection of *Leptospira* spp. in Urine

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We developed a new loop-mediated isothermal amplification (LAMP) method to detect *rrs*, a 16S rRNA gene of pathogenic *Leptospira* spp. in urine. The method enables detection of two leptospiral cells per reaction mixture following boiling of urine specimens. The sensitivity of this method is higher than that of culture or of *flaB* nested PCR.

Leptospirosis is a worldwide zoonosis caused by infection with pathogenic spirochetes of the genus *Leptospira*. *Leptospira* spp. colonize the proximal renal tubules of maintenance hosts, natural carrier animals of a particular leptospiral serovar, and are excreted in the urine. Transmission of leptospirosis in humans and nonmaintenance host animals occurs incidentally by exposure to water or soil contaminated by the urine of infected animals or by direct contact with infected animals. Leptospirosis is a significant public health problem in developing countries in the tropics, particularly Asia and Latin America (2, 11, 19). Its nonspecific and varied presentation in the early phase hampers clinical diagnosis and can lead to misdiagnosis as many other infectious diseases, including dengue fever or dengue hemorrhagic fever, malaria, and scrub typhus (9, 15, 20). Early diagnosis is essential because antibiotic treatment is most effective during the initial course of the disease (5, 21). Therefore, availability of a rapid and accurate point-of-care diagnostic test is required to identify leptospirosis; however, current diagnostic methods are not useful for early diagnosis (e.g., culture and microscopic agglutination test) or are not widely applicable in developing countries (e.g., PCR) (18).

Loop-mediated isothermal amplification (LAMP), unlike PCR, amplifies a target DNA sequence under isothermal conditions for approximately 1 h with high specificity and efficiency, and the results can be assessed with the naked eye (12). Thus, LAMP has potential applications as a diagnostic method in resource-limited countries. Two LAMP methods for leptospiral DNA detection have been published (10, 14). One method targets *lipL41* and detects leptospiral DNA by using purified DNA from mouse kidneys, but it has a detection limit of only l00 genome equivalents per reaction mixture (10). The other method comprises primers that target leptospiral *rrs*, a 16S rRNA gene (14). *rrs* LAMP and *lipL41* LAMP were evaluated using DNA samples extracted from sera of febrile patients. The results indicated that the specificity of *rrs* LAMP is lower than that of *lipL41* LAMP, and this hinders the clinical utility of *rrs* LAMP (14).

Leptospiral DNA has been detected by PCR during the chronic phase in urine of carrier animals and during the early phase in patients with leptospirosis (1, 3, 6, 13). Because urine collection is easy and less invasive than blood collection, we attempted to establish a new LAMP method, Lepto-*rrs* LAMP, which uses simplified sample processing to detect leptospiral DNA in urine.

Lepto-*rrs* LAMP primers were designed using PrimerExplorer V4 software (https://primerexplorer.jp/lamp4.0.0/index.html) and manually modified (see the methods described as well as Fig. S1 in the supplemental material). The reaction mixture (25 μl) for the Lepto-*rrs* LAMP contained 1.6 μM each primer (FIP, 5′-TAGTTTCAAGTGCAGGCTGCGAGGCGGACATGTAAGTCAGG-3′; BIP, 5′-GGAGTTTGGGAGAGGCAAGTGGGCCACTGGTGTTCCTCCA-3′; LF, 5′-GTTGAGCCCGCAGTTTTCAC-3′; LB, 5′-AATTCCAGGTGTAGCGGTGA-3′) and 0.2 μM other primers (F3, 5′-TCATTGGGCGTAAAGGGTG-3′; B3, 5′-AGTTTTAGGCCAGCAAGTCG-3′), in addition to 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 0.72 mM each deoxynucleotide triphosphate, 1 μl of a fluorescent detection reagent (Eiken Chemical Company, Tochigi, Japan), 8 U of *Bst* DNA polymerase (Lucigen, Middleton, WI), and 2 μl of DNA template. DNA templates were heated to 95°C for 2 min, followed by rapid cooling on ice before addition to the LAMP reaction mixture. LAMP reactions were performed at 65°C for 60 min, followed by termination at 95°C for 5 min using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Positive and negative results were distinguished by UV fluorescence (17). The Lepto-*rrs* LAMP primer set amplified the target sequences of all 14 pathogenic and intermediate *Leptospira* spp. (4). Conversely, none of the sequences of the six nonpathogenic *Leptospira* spp. or other bacterial species was amplified, even when 5 ng of purified DNA (10^6 genome equivalents) was used in each reaction mixture (Table 1). The lower detection limit when using purified DNA was determined to be 2 genome equivalents per reaction mixture under heat-denaturing conditions (see Fig. S2 in the supplemental

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TABLE 1 Bacteria used to determine the specificity of Lepto-*rrs* LAMP

Species (DNA group[a])	Serovar	Strain
L. alexanderi (P)	Yunnan	A 10
L. alstonii (P)	Pinchang	80-412
L. borgpetersenii (P)	Javanica	Veldrat Batavis 46
L. borgpetersenii (P)	Sejroe	M 84
L. interrogans (P)	Pomona	Pomona
L. interrogans (P)	Hardjo	Hardjoprajitno
L. interrogans (P)	Copenhageni	Fiocruz L1-130
L. kirschneri (P)	Grippotyphosa	Moskva V
L. kirschneri (P)	Cynopteri	3522 C
L. kmetyi (P)	Malaysia	Bejo-Iso
L. noguchii (P)	Panama	CZ 214
L. santarosai (P)	Shermani	1342 K
L. weilii (P)	Celledoni	Celledoni
L. broomii (I)	Undesignated	5399
L. fainei (I)	Hurstbridge	BUT 6
L. inadai (I)	Lyme	10
L. licerasiae (I)	Varillal	VAR 010
L. wolffii (I)	Korat	Korat-H2
L. biflexa (NP)	Patoc	Patoc I
L. meyeri (NP)	Semaranga	Veldrat Semarang 173
L. terpstrae (NP)	Hualin	LT 11-33
L. vanthielii (NP)	Holland	Waz Holland
L. wolbachii (NP)	Codice	CDC
L. yanagawae (NP)	Saopaulo	Sao Paulo
Borrelia burgdorferi		B31
Legionella pneumophila		Clinical isolate
Leptonema illini		3055
Neisseria gonorrhoeae		Clinical isolate
Staphylococcus aureus		Clinical isolate
Streptococcus pneumoniae		Clinical isolate
Turneriella parva		H
Uropathogenic *Escherichia coli*		Clinical isolate
Vibrio cholerae		Clinical isolate

[a] P, pathogenic species; I, intermediate species; NP, nonpathogenic species (as per 16S rRNA gene sequence analysis).

material) and 10 genome equivalents per reaction mixture under nondenaturing conditions (data not shown).

For the spiking assay, 1×10^8 *L. interrogans* serovar Pomona (strain Pomona) and *L. fainei* serovar Hurstbridge (strain BUT 6) cells, which were enumerated using a counting chamber of 0.010-mm depth (Nitirin, Tokyo, Japan) under dark-field microscopy, were centrifuged (4,000 × *g*, 15 min), resuspended in 1 ml of urine obtained from a healthy man, and then serially diluted 10-fold. The diluted samples were boiled for 10 min, and the supernatant was used as a template for Lepto-*rrs* LAMP. Positive results were obtained in samples of up to 10^3 cells/ml, indicating that the lower detection limit was 2 leptospiral cells per reaction mixture.

LAMP was then applied to detect leptospiral DNA from urine of carrier animals. First, Lepto-*rrs* LAMP and *flaB* nested PCR were performed using urine samples from Norway rats (*Rattus norvegicus*), whose kidney tissues were then cultured. The Norway rats were captured using live traps or sticky traps for vermin control in Tokyo. Voided urine was collected, following which the kidneys were excised under anesthesia and cultured in liquid modified Korthof's medium with 10% rabbit serum at 30°C, as described previously (7). The urine samples were processed using two procedures. In the first procedure, 20 μl of the urine sample was boiled for 10 min (boiled urine sample), whereas in the second procedure, 50 to 800 μl of the urine sample was centrifuged (16,000 × *g*, 10 min) and the resulting pellet was resuspended in 20 μl of 10 mM Tris-1 mM EDTA (TE; pH 8.0) and then boiled (urine pellet samples). The supernatant of the boiled sample was used as a template for Lepto-*rrs* LAMP and *flaB* nested PCR. *flaB* nested PCR was performed using previously described primers and conditions (8) with minor modifications: the reaction volume was 20 μl and contained 2 μl and 1 μl of the DNA template in the first and second PCRs, respectively. Lepto-*rrs* LAMP detected leptospiral DNA in 11 of 12 culture-positive boiled urine samples and 10 of 11 culture-positive urine pellet samples. These results were superior to those of the *flaB* nested PCR (Table 2). Intermittent excretion as well as a variable number of leptospires in urine have been demonstrated in carrier animals (13, 16), which may contribute to the failure of *rrs* detection using the LAMP method in culture-positive urine samples. Thus, repetition of nucleic acid amplification tests is recommended when urine samples are used. In addition, it is also advisable to immediately process collected urine (preferably within 2 h) or make it alkaline, because survival of leptospires in voided acid urine is limited (5, 21). Lepto-*rrs* LAMP detected leptospiral DNA in two culture-negative samples. DNA sequencing of each product revealed that these sequences belonged to pathogenic *Leptospira* spp. (data not shown). It is generally accepted that culturing of leptospires is difficult and has low sensitivity (2). Furthermore, as shown in our previous study (7), Norway rats in Tokyo carry an extremely fastidious *L. interrogans* strain (*flaB* sequence type; see RnTKD-2 and RatST3 in Fig. S3 of the supplemental material). These facts strongly suggest that the sensitivity of Lepto-*rrs* LAMP is higher than that of culture, and its specificity is also higher than that obtained in this study (66.7% compared with that of culture, which is regarded as the gold standard).

Next, Lepto-*rrs* LAMP and *flaB* nested PCR were performed using urine samples from field rats captured in Tokyo (different

TABLE 2 Comparison of results from Lepto-*rrs* LAMP and *flaB* nested PCR using urine samples from Norway rats and cultures of Norway rat kidney tissues

	Boiled urine[a] ($n = 18$)				Urine pellet[b] ($n = 16$[c])			
	Lepto-*rrs* LAMP		*flaB* nested PCR		Lepto-*rrs* LAMP		*flaB* nested PCR	
Culture result	No. positive	No. negative	No. positive	No. negative	No. positive	No. negative	No. positive	No. negative
Positive	11	1	6	6	10	1	9	2
Negative	2	4	0	6	2	3	1	4

[a] Each urine sample (20 μl) was boiled for 10 min, and 2 μl of the boiled urine sample was then used as the template.
[b] Urine samples were centrifuged (16,000 ×*g*, 10 min), the resulting pellets were resuspended in 20 μl TE, boiled for 10 min, and 2 μl of the boiled urine sample was then used as the template.
[c] The volumes of 2 of the 18 urine samples were sufficient only for preparation of boiled urine samples.

TABLE 3 Detection of leptospiral DNA in urine samples from field rats and from farmed pigs and buffaloes using Lepto-*rrs* LAMP and *flaB* nested PCR

Test method	Norway rats (Japan)[a] ($n = 16$)		Pigs (Japan) ($n = 29$)		Buffaloes (Philippines) ($n = 51$)	
	Boiled urine[b]	Urine pellet[c]	Boiled urine	Urine pellet	Boiled urine	Urine pellet
Lepto-*rrs* LAMP	10	13	0	5	0	10
flaB nested PCR	0	8	0	5	0	2

[a] Country names in parentheses indicate the place where the urine samples were collected.
[b] Each urine sample (20 μl) was boiled for 10 min, and 2 μl of the boiled urine sample was then used as the template.
[c] Urine samples were centrifuged (16,000 × *g*, 10 min), the resulting pellets were resuspended in 20 μl TE, boiled for 10 min, and 2 μl of the boiled urine sample was then used as the template.

individuals from those in Table 2) and farmed pigs and buffaloes, which appeared healthy and had never been identified as carriers (Table 3). Urine samples (150 μl to 1.9 ml from rats, 2.0 ml from pigs, and 1.5 to 2.0 ml from buffaloes) were processed as described above. The numbers of positive samples detected by Lepto-*rrs* LAMP in boiled urine and urine pellets from rats and urine pellet samples from buffaloes were higher than that detected by *flaB* nested PCR. All the urine samples from pigs and boiled urine samples from buffaloes were found to be negative by both the methods. All the positive samples identified by Lepto-*rrs* LAMP were sequenced and found to belong to pathogenic *Leptospira* spp. (data not shown). All the negative samples identified by Lepto-*rrs* LAMP were also found to be negative by *flaB* nested PCR (data not shown). Based on *flaB* sequences, the *Leptospira* spp. identified in this study were *L. interrogans* and *L. borgpetersenii* (see Fig. S3 in the supplemental material).

We succeeded in detecting leptospiral DNA in carrier animals by using Lepto-*rrs* LAMP. In incidental infections, significant leptospiruria is infrequent at the early stage and is assumed to start during the second week of illness (5). Moreover, the *Leptospira* type (species and/or serovar) and infection dose influence the clinical outcome, which may result in excretion of varied numbers of leptospires in urine. However, leptospiral DNA has been detected by PCR in urine samples collected from patients in the acute phase with a higher sensitivity than that in blood samples (1, 3, 6). Although further clinical validation is required, we also detected leptospiral *rrs* in urine from humans and dogs with acute leptospirosis (data not shown), indicating the applicability of Lepto-*rrs* LAMP for early diagnosis of leptospirosis.

In conclusion, we developed a new LAMP method, Lepto-*rrs* LAMP, which utilizes a simple DNA preparation step, to detect pathogenic *Leptospira* spp. in urine. In contrast to previously developed LAMP methods that use purified DNA samples from kidney tissues or sera (10, 14), this method can amplify the target DNA without DNA purification; boiled urine or urine pellet samples are sufficient to prepare the DNA template. This LAMP method also demonstrated a better detection sensitivity limit (2 genome equivalents per reaction mixture) than those demonstrated by previous methods, i.e., 100 genome equivalents per reaction mixture (10) and 10 genome equivalents per reaction mixture (14). In addition, this method is faster than *flaB* nested PCR and more sensitive for testing clinical samples. Moreover, Lepto-*rrs* LAMP guarantees high performance at a low cost. It has the potential to be used not only as a screening test for epidemiological studies and management and control of farmed animals but also as a point-of-care test for diagnose of acute leptospirosis in resource-limited settings where leptospirosis is endemic.

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