Journal of Clinical Microbiology, Short-Form

# A new loop-mediated isothermal amplification method for rapid, simple, and sensitive detection of *Leptospira* spp. in urine

Running title: Detection of *Leptospira* spp. in urine by LAMP

Nobuo Koizumi,[1*,#] Chie Nakajima,[2#] Tunehito Harunari,[3] Tsutomu Tanikawa,[3] Toshihiro Tokiwa,[4] Eriko Uchimura,[5] Tokujiro Furuya,[6] Claro Niegos Mingala,[7] Marvin Ardeza Villanueva,[7] Makoto Ohnishi,[1] and Yasuhiko Suzuki[2]

[1]Department of Bacteriology, National Institute of Infectious Disease, Tokyo, Japan

[2]Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Hokkaido, Japan

[3]Ikari Cooperation, Chiba, Japan

[4]Section of Environmental Parasitology, Graduate School of Tokyo Medical and Dental University, Tokyo, Japan

[5]Kagoshima Central Livestock Hygiene Service Center, Kagoshima, Japan

[6]Pfizer Japan Inc., Tokyo, Japan

[7]Animal Health Unit, Philippine Carabao Center National Headquarters and Genepool, Nueva Ecija, Philippines

[#]These authors contributed equally to this study.

[*]Corresponding author

Mailing address: 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Phone: 81-3-5285-1111 (ex. 2224)

Fax: 81-3-5285-1163

E-mail: nkoizumi@nih.go.jp

**Abstract**

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 following boiling of urine specimens. The sensitivity of this method is higher than that of culture or of *flaB*-nested PCR.

**Text**

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 non-maintenance 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 non-specific 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 one hour 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 using purified DNA from mouse kidneys but has a detection limit of only l00 genome equivalents per reaction (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 during the chronic phase in urine of carrier animals and during the early phase in patients with leptospirosis by PCR (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 (available online at https://primerexplorer.jp/lamp4.0.0/index.html) and manually modified (Supplemental methods and Fig. S1). The reaction mixture (25 μl) of Lepto-*rrs* LAMP contained 1.6 μM of each primer (FIP, 5′-TAGTTTCAAGTGCAGGCTGCGAGGCGGACATGTAAGTCAGG-3′; BIP, 5′-GGAGTTTGGGAGAGGCAAGTGGGCCACTGGTGTTCCTCCA-3′; LF, 5′-GTTGAGCCCGCAGTTTTCAC-3′; and LB, 5′-AATTCCAGGTGTAGCGGTGA-3′) and 0.2 μM of other primers (F3, 5′-TCATTGGGCGTAAAGGGTG-3′; and 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 of each deoxynucleotide triphosphate, 1 μl of a fluorescent detection reagent (Eiken Chemical Company, Tochigi, Japan), 8 U of *Bst* DNA polymerase (Lucigen, Middleton, WI, USA), 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, USA). 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 non-pathogenic *Leptospira* spp. and other bacterial spp. were amplified even when 5 ng of purified DNA ($10^6$ genome equivalents) was used in each reaction (Table 1). The lower detection limit using purified DNA was determined to be two genome equivalents per reaction under heat-denaturing conditions (Fig. S2) and 10 genome equivalents per reaction under non-denaturing conditions (data not shown).

For the spiking assay, $1 \times 10^8$ *L. interrogans* serovar Pomona (strain Pomona) and *L. fainei* serovar Hurstbridge (strain BUT 6), which were enumerated using a counting chamber of 0.010-mm depth (Nitirin, Tokyo, Japan) under dark-field microscopy, were centrifuged (4000 ×*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 two leptospiral cells per reaction.

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 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, 50–800 μl of the urine sample was centrifuged (16,000 ×*g*, 10 min) and the resulting pellets were resuspended in 20 µl of 10 mM 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 first and second PCR, 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 *flaB*-nested PCR (Table 2). Intermittent excretion as well as a variable number of leptospires in urine has been demonstrated in carrier animals (13, 16), which may contribute to the failure of *rrs* detection using the LAMP method in the culture-positive urine sample. 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 culture 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). 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 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–1.9 ml from rats, 2.0 ml from pigs, and 1.5–2.0 ml from buffaloes) were processed as described above. The number of positives detected by Lepto-*rrs* LAMP in boiled urine and urine pellet samples from rats and urine pellet samples from buffaloes was 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* (Fig. S3).

We succeeded in detecting leptospiral DNA in carrier animals 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 variable number of leptospires in urine. However, leptospiral DNA has been detected in urine samples collected from patients in the acute phase by PCR 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 (two genome equivalents per reaction) than those demonstrated by previous methods, i.e., 100 genome equivalents per reaction (10) and 10 genome equivalents per reaction (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.

## Acknowledgments

We are grateful to Y Fukushima, M Muto, NS Abes, and CA Gutierrez for their technical assistance. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) for the Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University, a Health Sciences Research Grant-in-Aid for Emerging and Re-emerging Infectious Diseases (H21-Shinkou-Ippan-004) from the Ministry of Health, Labour, and Welfare of Japan to NK, and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and the Global Center of Excellence (GCOE) Program from MEXT to YS.

## References

1. **Bal, A. E., C. Gravekamp, R. A. Hartskeerl, J. de Meza-Brewster, H. Korver, and W. J. Terpstra.** 1994. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J. Clin. Microbiol. **32:**1894–1898.
2. **Bharti, A. R., J. E. Nally, J. N. Ricaldi, M. A. Matthias, M. M. Diaz, M. A. Lovett, P. N. Levett, R. H. Gilman, M. R. Willig, E. Gotuzzo, and J. M. Vinetz.** 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. **3:**57-71.

3. **Brown, P. D., C. Gravekamp, D. G. Carrington, H. van de Kemp, R. A. Hartskeerl, C. N. Edwards, C. O. R. Everard, W. J. Terpstra, and P. N. Levett.** 1995. Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. J. Med. Microbiol. **43:**110–114.

4. **Cerqueira, G. M., and M. Picardeau.** 2009. A century of *Leptospira* strain typing. Infect. Genet. Evol. **9:**760–768.

5. **Faine, S., B. Adler, C. Bolin, and P. Perolat.** 1999. *Leptospira* and Leptospirosis, 2nd ed. MediSci, Melbourne.

6. **Fonseca, C. A., M. M. G. Teixeira, E. C. Romero, F. M. Tengand, M. V. da Silvae, and M. A. Shikanai-Yasuda.** 2006. *Leptospira* DNA detection for the diagnosis of human leptospirosis. J. Infect. **52:**15–22.

7. **Koizumi, N., M. Muto, T. Tanikawa, H. Mizutani, Y. Sohmura, E. Hayashi, N. Akao, M. Hoshino, H. Kawabata, and H. Watanabe.** 2009. Human leptospirosis cases and prevalence of rats harboring *Leptospira interrogans* in urban areas of Tokyo, Japan. J. Med. Microbiol. **58:**1227–1230.

8. **Koizumi, N., M. Muto, S. Yamamoto, Y. Baba, M. Kudo, Y. Tamae, K. Shimomura, I. Takatori, A. Iwakiri, K. Ishikawa, H. Soma, and H. Watanabe.** 2008. Investigation of

reservoir animals of *Leptospira* in the northern part of Miyazaki Prefecture. Jpn. J. Infect. Dis. **61:**465–468.

9. **LaRocque, R. C., R. F. Breiman, M. D. Ari, R. E. Morey, F. A. Janan, J. M. Hayes, M. A. Hossain, W. A. Brooks, and P. N. Levett.** 2005. Leptospirosis during dengue outbreak, Bangladesh. Emerg. Infect. Dis. 11:766–769.

10. **Lin, X., Y. Chen, Y. Lu, J. Yan, and J. Yan.** 2009. Application of a loop-mediated isothermal amplification method for the detection of pathogenic *Leptospira*. Diagn. Microbiol. Infect. Dis. **63:**237–242.

11. **McBride, A. J. L., D. A. Athanazio, M. G. Reis, and A. I. Ko.** 2005. Leptospirosis. Curr. Opin. Infect. Dis. **18:**376–386.

12. **Mori, Y., and T. Notomi.** 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. **15:**62–69.

13. **Rojas, P., A. M. Monahan, S. Schuller, I. S. Miller, B. K. Markey, and J. E. Nally.** 2010. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. Eur. J. Clin. Microbiol. Infect. Dis. **29:**1305–1309.

14. **Sonthayanon, P., W. Chierakul, V. Wuthiekanun, J. Thaipadungpanit, T. Kalambaheti, S. Boonsilp, P. Amornchai, L. D. Smythe, D. Limmathurotsakul, N. P. Day, and S. J.**

**Peacock.** 2011. Accuracy of Loop-Mediated Isothermal Amplification for Diagnosis of Human Leptospirosis in Thailand. Am. J. Trop. Med. Hyg. **84:**614–620.

15. **Suputtamongkol, Y., K. Niwattayakul, C. Suttinont, K. Losuwanaluk, R. Limpaiboon, W. Chierakul, V. Wuthiekanun, and S. Triengrim, M. Chenchittikul, and N. J. White.** 2004. An open, randomized, controlled trial of penicillin, doxycycline, and cefotaxime for patients with severe leptospirosis. Clin. Infect. Dis. 39:1417–1424.
16. **Thiermann, A. B.** 1982. Experimental leptospiral infections in pregnant cattle with organisms of the Hebdomadis serogroup. Am. J. Vet. Res. 43:780–784.
17. **Tomita, N., Y. Mori, H. Kanda, and T. Notomi.** 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. **3:**877–882.
18. **Toyokawa, T., M. Ohnishi, and N. Koizumi.** 2011. Diagnosis of acute leptospirosis. Expert Rev. Anti. Infect. Ther. **9:**111–121.
19. **Victoriano, A. F. B., L. D. Smythe, N. Gloriani-Barzaga, L. L. Cavinta, T. Kasai, K. Limpakarnjanarat, B. L. Ong, G. Gongal, J. Hall, C. A. Coulombe, Y. Yanagihara, S. Yoshida, and B. Adler.** 2009. Leptospirosis in the Asia Pacific region. BMC Infect. Dis. **9:**147.

20. **Wongsrichanalai, C., C. K. Murray, M. Gray, R. S. Miller, P. McDaniel P, W. J. Liao, A. L. Pickard, and A. J. Magill.** 2003. Co-infection with malaria and leptospirosis. Am. J. Trop. Med. Hyg. **68:**583–585.

21. **World Health Organization**. 2003. Human leptospirosis: guidance for diagnosis, surveillance, and control. WHO, Geneva.

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

| Bacterium (DNA group[a]) | Serovar | Strain |
|---|---|---|
| *L. alexanderi* (P) | Yunnan | A 10 |
| *L. alstonii* (P) | Pinchang | 80-412 |
| *L. borgpetersenii* (P) | Javanica | Veldrat Batavis 46 |
| | Sejroe | M 84 |
| *L. interrogans* (P) | Pomona | Pomona |
| | Hardjo | Hardjoprajitno |
| | Copenhageni | Fiocruz L1-130 |
| *L. kirschneri* (P) | Grippotyphosa | Moskva V |
| | 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.

Table 2. Comparison of results from Lepto-*rrs* loop-mediated isothermal amplification (LAMP) and *flaB*-nested PCR using urine samples from Norway rats and cultures of Norway rat kidney tissues.

| Culture | Boiled urine[a] (N = 18[c]) | | | | Urine pellet[b] (N = 16[c]) | | | |
|---|---|---|---|---|---|---|---|---|
| | Lepto-*rrs* LAMP | | *flaB*-nested PCR | | Lepto-*rrs* LAMP | | *flaB*-nested PCR | |
| | + | − | + | − | + | − | + | − |
| + | 11/18[d] | 1/18 | 6/18 | 6/18 | 10/16 | 1/16 | 9/16 | 2/16 |
| − | 2/18 | 4/18 | 0/18 | 6/18 | 2/16 | 3/16 | 1/16 | 4/16 |

[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) and 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 volume of two of the 18 urine samples was sufficient only for preparation of boiled urine samples.

[d]No. of positives/no. of samples tested.

Table 3. Detection of leptospiral DNA in urine samples from field rats and from farmed pigs and buffaloes using Lepto-*rrs* loop-mediated isothermal amplification (LAMP) and *flaB*-nested PCR

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

[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) and 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.

[d]No. of positives/no. of samples tested.