

Evaluation of loop-mediated isothermal amplification method (LAMP) for pathogenic *Leptospira* spp. detection with leptospires isolation and real-time PCR

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ABSTRACT. Leptospirosis has been one of the worldwide zoonotic diseases caused by pathogenic *Leptospira* spp. Many molecular techniques have consecutively been developed to detect such pathogen including loop-mediated isothermal amplification method (LAMP). The objectives of this study were to evaluate the diagnostic accuracy of LAMP assay and real-time PCR using bacterial culture as the gold standard and to assess the agreement among these three tests using Cohen's kappa statistics. In total, 533 urine samples were collected from 266 beef and 267 dairy cattle reared in central region of Thailand. Sensitivity and specificity of LAMP were 96.8% (95% CI 81.5–99.8) and 97.0% (95% CI 94.9–98.2), respectively. The accuracy of LAMP (97.0%) was significantly higher than that of real-time PCR (91.9%) at 95% CI. With Cohen's kappa statistics, culture method and LAMP were substantially agreed with each other (77.4%), whereas real-time PCR only moderately agreed with culture (47.7%) and LAMP (45.3%), respectively. Consequently, LAMP was more effective than real-time PCR in detecting *Leptospira* spp. in the urine of cattle. Besides, LAMP had less cost and was simpler than real-time PCR. Thus, LAMP was an excellent alternative for routine surveillance of leptospirosis in cattle.

KEY WORDS: accuracy, cattle, LAMP, leptospirosis, urine

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Leptospirosis is one of the global zoonotic diseases caused by pathogenic *Leptospira* spp. [21]. Non-specific clinical signs were mainly described in both infected humans and animals [13, 20]. A number of animal species are considered reservoirs of leptospirosis, such as rodents, cattle, pigs, sheep and dogs [13]. These infected animals do not show any anomaly, but they are capable of shedding the leptospires via their urines into the environment. In addition, the pathogens can survive under suitable moist conditions for a certain period of time [15]. The gold standard for detecting *Leptospira* spp. is bacterial culture. However, this particular method is time-consuming and impractical for routine diagnosis, as well as crucially requires expertise [27]. Therefore, the faster, higher sensitivity and more practical method like polymerase chain reaction (PCR) becomes the most widely used for leptospires detection [19]. Nonetheless, other novel molecular techniques have consecutively been developed nowadays.

Loop-mediated isothermal amplification method (LAMP)

is one of those techniques. The LAMP assay was firstly developed by Notomi *et al.* This method is rapid, effective and highly specific under isothermal conditions [17]. The LAMP assay for the detection of several targeting genes of *Leptospira* spp. has been published. A combination of primer sets targeting *lipL32* and *lipL41* genes for *Leptospira* spp. detection of human specimens, shows positive results with PCR *Leptospira*-positive samples which are classified into three species including *L. interrogans*, *L. borgpetersenii* and *L. weilii* [5]. The study of Koizumi *et al.* developing the LAMP method to detect *rrs*, a 16S ribosomal RNA gene of pathogenic *Leptospira* spp. in urine of carrier animals [10] indicates that these primer sets determined eight pathogenic (*L. interrogans*, *L. kirshneri*, *L. borgpetersenii*, *L. santarosai*, *L. alexanderi*, *L. noguchii*, *L. weilii* and *L. alstonii*) and five intermediate *Leptospira* spp. (*L. broomii*, *L. fainei*, *L. inadai*, *L. licerasiae* and *L. wolffii*). Conversely, nonpathogenic *Leptospira* spp. and other bacterial species show negative results [10]. Compared to PCR-based methods, LAMP has the advantages of reaction simplicity and higher amplification efficiency [18]. Moreover, the reaction can be observed by naked eyes with visual fluorescence [18].

Due to its simplicity, LAMP is highly applicable for the limited resourced countries. In Thailand, the Animal Leptospirosis Center, National Institute of Animal Health, Department of Livestock Development has applied this technique to detect 16S rDNA of pathogenic leptospires in animals, mainly cattle, living in the country. To practically apply this assay in the field, sensitivity and specificity of the test are highly recommended to be evaluated. Besides, the agree-

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ment with other methods like PCR should also be considered. Thus, the objective of the present study was to evaluate the diagnostic accuracy of LAMP assay and real-time PCR using bacterial culture as the gold standard.

MATERIALS AND METHODS

Sample collection: 20 ml of fresh urine was collected from individual cattle in Lopburi, Chainat and Nakhon Nayok provinces in the central region of Thailand from October 2012 to February 2014 for the propose of disease surveillance. After collection, the samples were kept in cool storage (4°C) and transported to the National Institute of Animal Health. All samples were subsequently examined with three methods including bacterial culture, real-time PCR and LAMP assay.

Leptospira spp. Culture: One ml of urine samples was added to 9 ml of 1% bovine serum albumin diluent with 5 Fluro-uracil transport media and was delivered immediately to the laboratory for bacterial isolation. The samples were cultured in EMJH (Johnson and Harris modification of the Ellinghausen and McCullough medium) semi-solid media with 5 Fluro-uracil (Becton-Dickinson Biosciences, Detroit, MI, U.S.A.) at 30°C for 12–16 weeks. *Leptospira* spp. were considered positive by the characteristic of thin helical structures with prominent hooked ends and motility under a dark field microscope [22]. The suspected samples were further subcultured in the EMJH liquid media and purified with 0.2 µm-pore-size membrane filter to remove the contaminants.

DNA extraction: DNA in 10 ml of urine sample was extracted using Dynabeads® DNA DIRECT™ Universal Kit (Invitrogen, Oslo, Norway) according to the manufacturer's instructions; DNA concentration was measured by Qubit® Fluorometer (Invitrogen).

Loop-mediated isothermal amplification method (LAMP): The LAMP was carried out using primer and condition previously described in [24]. This method used 10 ng of extracted DNA. Briefly, a total of 25 µl reaction mixture contained 1.0 µM outer primers F3 (5'-GAACCTGAAGCAGC-GAC-3') and B3 (5'-GACTTACATGTCCGCTACG-3'), 1.6 µM inner primers FIP (5'-CACATCGCTGCT-TATTTTCCCTGCGGTGAACGATGAAGGTCT-3') and BIP (5'-CCTGCCTAAAGCACCGGCTAACGCCCAAT-GATTCCGAACAA-3'), 0.8 µM loop primer LB (5'-AGCC-GCGGTAATACGTATGGTG-3'), 1X Thermopol® reaction buffer (New England Biolabs, Hitchin, U.K.), 4 mM MgSO₄ (New England Biolabs), 1 M Betaine (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.4 mM dNTPs (New England Biolabs), 0.5 mM MnCl₂, 25 µM Calcein and 8 units *Bst* DNA polymerase (New England Biolabs) and adjusted to a total volume of 25 µl with sterile water (New England Biolabs). The reaction mixture was incubated at 61°C for 90–120 min in a heating block and then was heated at 80°C for 2 min to terminate the reaction. The presence of product was defined on the basis of green color detected with naked eyes.

Real-time PCR: Real-time PCR was conducted according to [23] by targeting the 241 bp region of *lipL32* gene which encoded the major outer membrane of pathogenic *Leptospira*

spp. [8, 9]. The Light Cycler® NanoInstrument (Roche Diagnostic, Mannheim, Germany) was used for hydrolysis probe assay. The DNA product was amplified using FastStartTaq DNA Polymerase (Roche Diagnostic) with the primers LipL32-45F (5'-AAGCATTACCGCTTGTGGTG-3'), LipL32-286R (5'-GAACTCCCATTTCAGCGATT-3') and Probe-189P ([FAM] 5'-AAAGCCAGGACAAGCGCCG-3'[BHQ-1]). The reaction mixture consisted of 10 ng of extracted DNA, 0.25 µM each primers and probe and 1X FastStart essential DNA probe master and was adjusted to a total volume of 20 µl with sterile water (Roche Diagnostic). The thermal condition of a holding stage was performed at 95°C for 10 min and followed by 45 cycles of two-step amplification from 95°C to 55°C for 30 sec.

Statistical analysis: Bacterial culture was considered the gold standard for *Leptospira* spp. detection in this study [26]. Sensitivity and specificity of LAMP and real-time PCR were calculated following [2]; accuracy of these tests was calculated according to the formulas proposed in [6]. The formulas described in [3] were used in positive and negative predictive values calculation. Equations 1–7 were used in these analyses;

Percent positivity; $PP = (TP + FP) / (TP + FP + TN + FN)$ (1)

Sensitivity; $SE = TP / (TP + FN)$ (2)

Specificity; $SP = TN / (TN + FP)$ (3)

Gold Standard Prevalence; $GSP = (TP + FN) / (TP + FP + TN + FN)$ (4)

Positive Predictive Value; PPV

$= (SE \times GSP) / ((SE \times GSP) + ((1 - SP) \times (1 - GSP)))$ (5)

Negative Predictive Value; NPV

$= (SP \times (1 - GSP)) / (((1 - SE) \times GSP) + ((SP) \times (1 - GSP)))$ (6)

Accuracy $= (TP + TN) / (TP + FP + TN + FN)$ (7)

, where TP, FP, TN and FN refer to true positive, false positive, true negative and false negative, respectively.

Cohen's kappa statistics for agreement of all the tests were analyzed, and the strength of agreement was interpreted as proposed in [12].

$K = (P_o - P_e) / (1 - P_e)$ (8)

, where K, P_o and P_e represent Cohen's kappa coefficient, relative observed agreement of the tests and hypothetical probability of agreement, respectively.

All statistical analyses were carried out with Program R version 3.2.2 (R development Core Team, Vienna, Austria). Algorithms in package 'caret' were used in the calculation of SE, SP, PPV and NPV [11], whereas Cohen's kappa statistics were performed within the package 'fmsb' [16].

RESULTS

Prevalence: A total of 533 urine samples were collected from 266 beef and 267 dairy cattle. It was found that 31, 45 and 58 animals were positive from Leptospiral culture, LAMP and real-time PCR, respectively. Thus, the prevalence of *Leptospira* spp. detection from these three assays was 5.8% (Confidence Interval (CI) 4.0–8.2), 8.4% (95% CI 6.2–11.1) and 10.9% (95% CI 8.4–13.8), respectively.

Accuracy: LAMP and real-time PCR were evaluated using bacterial culture as a gold standard. The examination results

Table 1. Comparison of examination results from LAMP and real-time PCR with culture method in the detection of *Leptospira* spp. in urine samples collected from cattle in Central region of Thailand

| Culture result | LAMP | | Real-time PCR | |
|----------------|--------------|--------------|---------------|--------------|
| | No. positive | No. negative | No. positive | No. negative |
| No. positive | 30 | 1 | 23 | 8 |
| No. negative | 15 | 487 | 35 | 467 |

comparing bacterial culture with two diagnostic assays are illustrated in Table 1.

LAMP was more sensitive and more specific than real-time PCR as could be obviously seen that the sensitivity of LAMP and real-time PCR was 96.8% (95% CI 81.5–99.8) and 74.2% (95% CI 55.1–87.5), while the specificity of LAMP and real-time PCR was 97.0% (95% CI 94.9–98.2) and 93.0% (95% CI 90.3–95.0), respectively. As for the accuracy, that of LAMP (97.0%; 95% CI 95.2–98.3) was significantly higher than that of real-time PCR (91.9%; 89.3–94.1). Both assays had higher negative predictive values (LAMP: 99.8%; 95% CI 98.7–100.0 and real-time PCR: 98.3%; 95% CI 96.6–99.2) than the positive ones (LAMP: 66.7%; 95% CI 50.9–79.6 and real-time PCR: 39.7%; 95% CI 27.3–53.4). Comparing these two assays, higher positive and negative predictive values were observed in LAMP assay.

Agreement of the tests: To further assess the strength of agreement among all studied assays, Cohen's kappa statistics were then analyzed. Culture method and LAMP substantially agreed with each other (77.4%; 95% CI 66.5–88.3), whereas real-time PCR only moderately agreed with culture (47.7%; 95% CI 32.7–62.7) and LAMP (45.3%; 95% CI 31.0–59.6).

DISCUSSION

The present study evaluated the diagnostic accuracy of two rapid molecular methods including LAMP and real-time PCR, in the detection of *Leptospira* genetic materials in urine samples from cattle in central region of Thailand. In this study, bacterial culture was determined as the gold standard and used for accuracy assessment. Thus, the prevalence derived from culture was considered a 'true prevalence.' Comparing the true prevalence with the prevalence derived from LAMP and real-time PCR (Table 1.), overestimated prevalence was found in both assays, especially from real-time PCR (10.9%; 95% CI 8.4–13.8); it was almost two times higher than the true prevalence (5.8%; 95% CI 4.0–8.2), whereas that of LAMP fell in the middle at 8.4% (95% CI 6.2–11.1). Our results corresponded with the previous study [7], suggesting that positivity in molecular techniques could be obtained with no positive culture results.

Regarding diagnostic accuracy of LAMP and real-time PCR, LAMP was more accurate method in the detection of *Leptospira* spp. with higher sensitivity and specificity than real-time PCR. The LAMP assay targeting 16s rDNA gene used in the present study had a low detection limit at 10–100 copies [24]. Therefore, its sensitivity was high as it could detect the pathogen even with low amount of genetic materials

[10]. In contrast, it was found that real-time PCR targeting *lipL32* gene was a low sensitive method with the sensitivity of 43% (95% CI 34–52%) [25], and the detection limit of real-time PCR was found at 100–1,000 copies per reaction under the author's conditions. The lower sensitivity of real-time PCR, compared to LAMP, was attributed to the difference in lower detection limits of the two methods. However, the low sensitivity of real-time PCR in this study also resulted from the high number of false negative samples (25.8%; 8/31) which were negative with real-time PCR but positive with culture. This poorly understood phenomenon was previously observed in a previous study [25]. The plausible explanation was that a very low count in the original sample might stochastically present in the aliquot prepared for culture but not in that for real-time PCR [25]. In terms of specificity, both LAMP and real-time PCR were considered a highly specific assay which was consistent with other previous publications [4, 10, 24, 25]. The primers used in the present study were preliminarily tested with some intermediate species of leptospires and demonstrated some mismatched characteristics. However, the test did not guarantee that the primer set never amplifies the target gene from any other intermediate species. To prove this, an experimental work is suggested in the further study. The possible amplification of target gene from intermediate species by the LAMP primer set may contribute to higher sensitivity of LAMP than real-time PCR.

Apart from sensitivity and specificity, predictive values changed proportional to the prevalence changes [18]. The predictive values gave a basic idea on how we can predict the results using a specific test with a specific population in a specific time. In this study, NPV was high in both LAMP (99.8%; 95% CI 98.7–100.0%) and real-time PCR (98.3%; 95% CI 96.6–99.2%). Hence, the uninfected animals trended to be tested negative. In contrast, the PPV of these two tests was moderate to low (66.7% and 39.7% for LAMP and real-time PCR, respectively). The infected individuals might not be well predicted by the positive results. However, as the predictive values depended largely on prevalence, the values were not transferrable from one to another study with different spaces and times.

Based on the Cohen's kappa statistics calculated in this study, real-time PCR only moderately agreed with the LAMP and the culture. This result was in agreement with the previous studies providing a notice on the possible disagreement of real-time PCR and culture [7, 25]. Moreover, LAMP was much more sensitive and slightly more specific than real-time PCR. Therefore, the agreement of these two tests was only at moderate level.

Bacterial culture which was considered the gold standard in the present study was regarded as a technique with low sensitivity, laborious, time consuming [7] and not practical in the early detection of leptospires [26]. To confront with this problem, various molecular techniques have been employed to rapidly identify the pathogens including real-time PCR and LAMP. However, real-time PCR was challenging in term of finance, technical and instrumental requirements and might not be applicable in developing countries where leptospirosis has been endemic [1, 26]. Therefore, the less

expensive method with less expertise requirement, such as LAMP, has been developed and implemented for the early detection of *Leptospira* pathogen [14]. As demonstrated in our study, LAMP was more effective than real-time PCR in the detection of pathogenic *Leptospira* spp. in the urine of cattle with substantially less cost and more simplicity. Accordingly, LAMP was an excellent alternative for routine surveillance of leptospirosis in cattle.

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REFERENCES

- Ahmed, A., van der Linden, H. and Hartskeerl, R. A. 2014. Development of a recombinase polymerase amplification assay for the detection of pathogenic *Leptospira*. *Int. J. Environ. Res. Public Health* **11**: 4953–4964. [Medline] [CrossRef]
- Altman, D. G. and Bland, J. M. 1994a. Diagnostic tests. 1: Sensitivity and specificity. *BMJ* **308**: 1552. [Medline] [CrossRef]
- Altman, D. G. and Bland, J. M. 1994b. Diagnostic tests 2: Predictive values. *BMJ* **309**: 102. [Medline] [CrossRef]
- Cermakova, Z., Kucerova, P., Pliskova, L. and Kubickova, P. 2013. Real-time PCR method for the detection of the gene encoding surface lipoprotein LipL32 of pathogenic *Leptospira*: use in the laboratory diagnosis of the acute form of leptospirosis. *Scand. J. Infect. Dis.* **45**: 593–599. [Medline] [CrossRef]
- Chen, H. W., Weissenberger, G., Atkins, E., Chao, C. C., Suptamongkol, Y. and Ching, W.M. 2015. Highly Sensitive Loop-Mediated Isothermal Amplification for the Detection of *Leptospira*. *Int. J. Bacteriol.* **1**: 1–6.
- Eusebi, P. 2013. Diagnostic accuracy measures. *Cerebrovasc. Dis.* **36**: 267–272. [Medline] [CrossRef]
- Fornazari, F., da Silva, R. C., Richini-Pereira, V. B., Beserra, H. E., Luvizotto, M. C. and Langoni, H. 2012. Comparison of conventional PCR, quantitative PCR, bacteriological culture and the Warthin Starry technique to detect *Leptospira* spp. in kidney and liver samples from naturally infected sheep from Brazil. *J. Microbiol. Methods* **90**: 321–326. [Medline] [CrossRef]
- Haake, D. A., Chao, G., Zuerner, R. L., Barnett, J. K., Barnett, D., Mazel, M., Matsunaga, J., Levett, P. N. and Bolin, C. A. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect. Immun.* **68**: 2276–2285. [Medline] [CrossRef]
- Hauk, P., Macedo, F., Romero, E. C., Vasconcellos, S. A., de Moraes, Z. M., Barbosa, A. S. and Ho, P. L. 2008. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect. Immun.* **76**: 2642–2650. [Medline] [CrossRef]
- Koizumi, N., Nakajima, C., Harunari, T., Tanikawa, T., Tokiwa, T., Uchimura, E., Furuya, T., Mingala, C. N., Villanueva, M. A., Ohnishi, M. and Suzuki, Y. 2012. A new loop-mediated isothermal amplification method for rapid, simple, and sensitive detection of *Leptospira* spp. in urine. *J. Clin. Microbiol.* **50**: 2072–2074. [Medline] [CrossRef]
- Kuhn, M. 2008. Building Predictive Models in R Using the caret Package. *J. Stat. Softw.* **28**: 1–26. [CrossRef]
- Landis, J. R. and Koch, G. G. 1977. The measurement of observer agreement for categorical data. *Biometrics* **33**: 159–174. [Medline] [CrossRef]
- Levett, P. N. 2001. Leptospirosis. *Clin. Microbiol. Rev.* **14**: 296–326. [Medline] [CrossRef]
- Lin, X., Chen, Y., Lu, Y., Yan, J. and Yan, J. 2009. Application of a loop-mediated isothermal amplification method for the detection of pathogenic *Leptospira*. *Diagn. Microbiol. Infect. Dis.* **63**: 237–242. [Medline] [CrossRef]
- Monahan, A. M., Callanan, J. J. and Nally, J. E. 2008. Proteomic analysis of *Leptospira interrogans* shed in urine of chronically infected hosts. *Infect. Immun.* **76**: 4952–4958. [Medline] [CrossRef]
- Nakazawa, M. 2015. Package fmsb. <https://cran.r-project.org/web/packages/fmsb/fmsb.pdf>. (accessed 15 December 2015).
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **28**: E63. [Medline] [CrossRef]
- Parida, M., Sannarangaiah, S., Dash, P. K., Rao, P. V. and Morita, K. 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev. Med. Virol.* **18**: 407–421. [Medline] [CrossRef]
- Picardeau, M., Bertherat, E., Jancloes, M., Skouloudis, A. N., Durski, K. and Hartskeerl, R. A. 2014. Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies. *Diagn. Microbiol. Infect. Dis.* **78**: 1–8. [Medline] [CrossRef]
- Rajeev, S., Ilha, M., Woldemeskel, M., Berghaus, R. D. and Pence, M. E. 2014. Detection of asymptomatic renal *Leptospira* infection in abattoir slaughtered cattle in southeastern Georgia, United States. *SAGE Open Medicine* **2**.
- Saito, M., Miyahara, S., Villanueva, S. Y., Aramaki, N., Ikejiri, M., Kobayashi, Y., Guevarra, J. P., Masuzawa, T., Gloriani, N. G., Yanagihara, Y. and Yoshida, S. 2014. PCR and culture identification of pathogenic *Leptospira* spp. from coastal soil in Leyte, Philippines, after a storm surge during Super Typhoon Haiyan (Yolanda). *Appl. Environ. Microbiol.* **80**: 6926–6932. [Medline] [CrossRef]
- Saito, M., Villanueva, S. Y., Chakraborty, A., Miyahara, S., Segawa, T., Asoh, T., Ozuru, R., Gloriani, N. G., Yanagihara, Y. and Yoshida, S. 2013. Comparative analysis of *Leptospira* strains isolated from environmental soil and water in the Philippines and Japan. *Appl. Environ. Microbiol.* **79**: 601–609. [Medline] [CrossRef]
- Stoddard, R. A. 2013. Detection of pathogenic *Leptospira* spp. through real-time PCR (qPCR) targeting the LipL32 gene. *Methods Mol. Biol.* **943**: 257–266. [Medline] [CrossRef]
- Suwancharoen, D., Kulchim, C., Chirathaworn, C. and Yoshida, S. 2012. Development of a novel primer combination to detect pathogenic *Leptospira* by loop-mediated isothermal amplification. *J. Microbiol. Methods* **91**: 171–173. [Medline] [CrossRef]
- Thaipadungpanit, J., Chierakul, W., Wuthiekanun, V., Limmathurotsakul, D., Amornchai, P., Boonslip, S., Smythe, L. D., Limpiboon, R., Hoffmaster, A. R., Day, N. P. and Peacock, S. J. 2011. Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipL32 genes for human leptospirosis in Thailand: a case-control study. *PLoS ONE* **6**: e16236. [Medline] [CrossRef]
- Toyokawa, T., Ohnishi, M. and Koizumi, N. 2011. Diagnosis of acute leptospirosis. *Expert Rev. Anti Infect. Ther.* **9**: 111–121. [Medline] [CrossRef]
- World Health Organization. 2003. Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control. *WHO Library* **45**: 1–109.