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ORIGINAL ARTICLE

Evaluation of LAMP for detection and/or screening of *Leptospira* spp. infection among domestic animals in the Philippines

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ABSTRACT

Objective: This study assessed the applicability of loop-mediated isothermal amplification (LAMP) for the detection of leptospirosis among domesticated animals and sewage rats. Specifically, it evaluated the ability of LAMP to amplify *Leptospira* spp. targeting the 16s rRNA gene in boiled urine samples.

Materials and methods: A total of 140 samples from different domestic animals were tested for the presence of the antigen. A nested-polymerase chain reaction (nPCR) protocol was used to compare and determine the sensitivity of LAMP in detecting *Leptospira* spp. The LAMP was also evaluated by comparing its amplification result using agarose gel electrophoresis and color change using dye.

Results: Positivity rate of *Leptospira* spp. antigen was 29.0% (40/140) for LAMP and 9.3% (13/140) for nPCR. Also, LAMP results for gel electrophoresis and dye color change varied in some samples that may be due to the interpretation of the result in dye color change.

Conclusion: Overall, LAMP is a rapid, sensitive, and cost-effective diagnostic method compared with nPCR. Also, LAMP has a potential application as pen-side screening, surveillance, and clinical diagnostic kits of infectious diseases without requiring advance equipment and skilled personnel.

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Introduction

Leptospirosis is an emerging communicable disease, an under-diagnosed disease, with major health effect, particularly in the developing countries [1,2]. It is a disease caused by the zoonotic pathogenic members of the genus *Leptospira*, a spirochete bacterium [3,4,5].

The accounts on the incidence of leptospirosis during the past 5 years in the Philippines are quite alarming. An increase of 62.35% in leptospirosis from the year 2011 to 2012 alone was reported in this country [6]. Leptospirosis is a zoonotic disease that can be communicated either directly between hosts to humans or indirectly from the environment. The organism usually enters the body through mucous membranes or abraded skin [4,7]. In densely populated areas in the country, rats usually transmit *Leptospira* spp. through excretion in the urine.

Leptospirosis has varying and non-specific clinical presentation that can lead to misdiagnosis as other infectious disease [8,9]. In addition, infected animals serve as carriers harboring leptospires in the kidneys and sporadically sheds the organism into the environment for a long period of time [10]. Fast initial diagnosis is vital because antimicrobial therapy is only an effective treatment in the early stages of the disease [4]. Thus, an accurate and precise pen-side diagnostic test kit is necessary to classify the disease as leptospirosis though bacterial culture, antibody detection methods (e.g., MAT or ELISA), and molecular test (e.g., PCR) are not suitable for early diagnosis and not usually applicable in most developing countries [11].

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Loop-mediated isothermal amplification (LAMP) is a rapid, sensitive, and economical molecular diagnostic test that can aid as a substitute diagnostic test for the detection of *Leptospira* spp. [12]. In this investigation, we evaluated LAMP in the detection of *Leptospira* spp. in boiled urine samples coming from different animals. Particularly, we compared the efficiency of LAMP and polymerase chain reaction (PCR) assay in the detection of *Leptospira* spp. The LAMP was also evaluated by comparing its amplification result using agarose gel electrophoresis and color change using dye. This is particularly important in the early and sensitive diagnosis of leptospirosis and in management and control of the pathogenic agent as well.

Materials and Methods

Sample collection and DNA extraction

Twenty urine samples were collected from representatives of each animal species (dogs, cats, pigs, goats, buffaloes, cattle, and sewage rats). The samples were collected from animals suspected with leptospirosis based on the clinical signs observed and history of infection within the herd or area. All urine samples were collected in Nueva Ecija, except for cattle samples collected in Laguna, Philippines. Before sample collection, a consent form was given to the farm and pet owners and veterinarians, respectively. Sewage rats were collected from the local market and rice field. Clean catch method was used in large animals while catheterization was performed to collect a urine sample in companion animals. For sewage rats, after the animals were euthanized performed following the standard euthanasia guidelines and with a supervision of a licensed veterinarian, direct collection of urine from the urinary bladder was performed.

About 200 ml of urine from each animal was taken and placed in a sterile 1.5 ml microcentrifuge tubes. The tubes containing the samples were centrifuged at 15,000 at 4°C rpm for 10 min. After which, the supernatant was discarded. Tris-EDTA buffer was added into the tube and the mixture was mixed briefly and heated at 95°C for 10 min in a heat block. The tubes containing the DNA samples were kept at 4°C until used. DNA from Leptoferm-C1® (Pfizer, New York) vaccine containing killed *Leptospira* spp. was also extracted and used as a positive control during the experiment.

Amplification of β-actin gene

A housekeeping gene, β-actin gene, was used to evaluate the quality of the extracted DNA. The PCR proceeded with total volume of 18.1 ml comprising 12.5 ml Top Taq (Qiagen, Germany), 1.6 ml 25 mM $MgCl_2$ (Promega, Madison, WI), 0.5 ml of each of 20 pmol forward and reverse primers (Table 1), and 3.0 ml of extracted DNA. Conditions for the thermal cycling were as follows: initial denaturation of protein for 5 min at 94°C, 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, extension for 30 sec for 72°C, and final extension step for 5 min at 72°C to complete the reaction. The expected amplicon size of this gene is 227 base pairs [13]. Only the samples that are positive for the β-actin gene were used for the next procedures.

Leptospira *spp. detection using LAMP*

Previously published primers for LAMP [14] were used for *Leptospira* spp. detection (Table 1). These designed primers were used to detect the *Leptospira* spp 16s rRNA gene. Twenty randomly selected samples from each animal commodity were used in the detection of *Leptospira* spp. The LAMP mixture was carried out in a total quantity of 12.5 ml: 4.5 ml sterile double distilled

Table 1. Primers used in the amplification of B-actin gene and *Leptospira* spp.

Primer	Sequence	Reference
B-actin F	5′-CGC ACC ACC GGC ATC GTG AT-3′	[13]
B-actin R	5′-TCC AGG GCC ACG TAG CAG AG-3′	
Lept-F3	5′-TCATTGGGCGTAAAGGGTG-3′	[14]
Lept-B3	5′-AGTTTTAGGCCAGCAAGTCG-3′	
Lept-FIP	5′-TAGTTTCAAGTGCAGGCTGCGAGGCGGACATGTAAGTCAGG-3′	
Lept-BIP	5′-GGAGTTTGGGAGAGGCAAGTGGGCCACTGGTGTTCCTCCA-3′	
Lept-F-Loop	5′-GTTGAGCCCGCAGTTTTCAC-3′	
Lept-B-Loop	5′-AATTCCAGGTGTAGCGGTGA-3′	
Lept-*flab* F1	5′-CTAACCGTTCTCTAAAGTTCAAC-3′	[15]
Lept-*flab* R1	5′-TGAATTCGGTTTCATATTTGCC-3′	
Lept-*flab* F2	5′-TGTGCACAAGACGATGAAAGC-3′	
Lept-*flab* R2	5′-AACATTGCCGTACCACTCTG-3′	

water, 5 M 3.0 ml of Betaine (Sigma, Germany), and 1.5 ml of 10× Buffer (Lucigen, Middleton, WI) which consisting of 20 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, and 1% Triton X-100, 0.2 mM each dNTPs (Intron), 0.75 ml of primer mix comprising 3.846 pmol apiece of the outer primers, 30.769 pmol apiece of inner primers, 15.38 pmol apiece of loop primers, 0.75 ml of 8,000 U/ml Bst Polymerase (Lucigen), and 1.0 ml of extracted DNA. The LAMP mixture was incubated 65°C for 1 h in a heat block. The tubes containing the LAMP mixture was placed on ice for 3 min before the addition of 0.5 ml SYBR Green dye (Invitrogen, Waltham, MA). The LAMP product was also electrophoresed using 2% agarose gel stained with Gel Red (Biotium, Fremont, CA). A multiple ladder-like bands pattern on gel electrophoresis of the LAMP products indicates positive sample. Change of color from orange to green can also be observed from samples positive for *Leptospira* spp., and no color change will be observed for negative samples (Fig. 1). The result of LAMP after addition of dye and agarose gel electrophoresis was also compared to determine which parameter or method is more reliable in the interpretation of LAMP result.

Detection of Leptospira *spp. by nPCR*

To cross check the efficiency of LAMP in detecting *Leptospira* spp., the same samples from each domestic animal were also subjected to nPCR based on previously published primers targeting the Leptospira-*flab* gene [15]. The nPCR mixture has a total quantity of 10.0 ml comprising 2.0 ml of 5× PCR buffer (Promega, Madison, WI), 1.4 ml of 25 mM $MgCl_2$ (Promega, Madison, WI), 0.5 ml of 2.5 mM each dNTP (Intron, Korea), 0.1 ml of Taq polymerase (FlexiTaq, Promega, Promega, Madison, WI), 1.5 ml of the extracted DNA and sterile double distilled water and 0.3 ml of each of the 10 mm forward and reverse first nPCR primers (Table 1). Thermal cycling conditions for the first PCR were as follows: initial denaturation at 95°C for 25 sec, followed by 25 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 7 min. For the second PCR, 3.0 ml of the first PCR product was used as a template and the amplification proceeded following the above-mentioned PCR protocols except for the primers using the second nPCR primers set (Table 1). On 2% agarose gel stained with GelRed (Biotium), the PCR product was electrophoresed. A positive sample indicating pathogenic *Leptospira* spp. has an amplicon size of 730 bp, while non-pathogenic have a 400 bp band on an agarose gel (Fig. 2) [15].

Data analysis

The positivity rate (%) of *Leptospira* spp. infection was computed by dividing the total number of positive samples by the total number of samples per animal commodity and multiplied by 100. Efficiency of LAMP method in the detection of *Leptospira* spp. was determined by comparing its result with that of the nPCR. Results were computed according to an animal commodity.

Results

Infection with the spirochete bacterium *Leptospira* spp. is becoming a widespread problem, especially in tropical countries. We evaluated LAMP as a fast, efficient, and cost-effective method in the detection of *Leptospira* spp. among the domestic animal. This is particularly important since these animals are the carriers and reservoir hosts of the microorganism. An effective control and

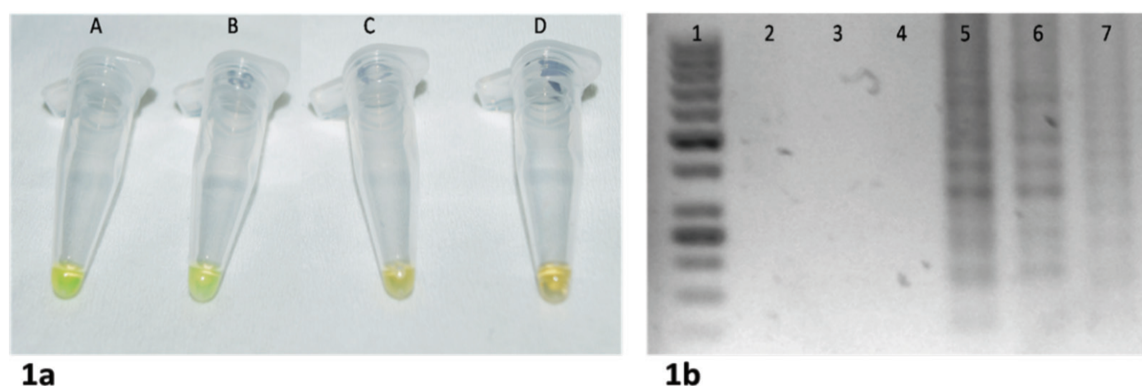

Figure 1. Evaluation of LAMP result using SYBR Green dye and agarose gel electrophoresis. (a) The result of LAMP after addition of SYBR Green dye, wherein positive samples are indicated by green coloration (Tubes A and B) and orange coloration for negative samples (Tubes C and D). (b) The characteristic ladder-like bands pattern in positive samples after LAMP (Lanes 5, 6, and 7). Lane 1 is a 100-bp DNA Ladder.

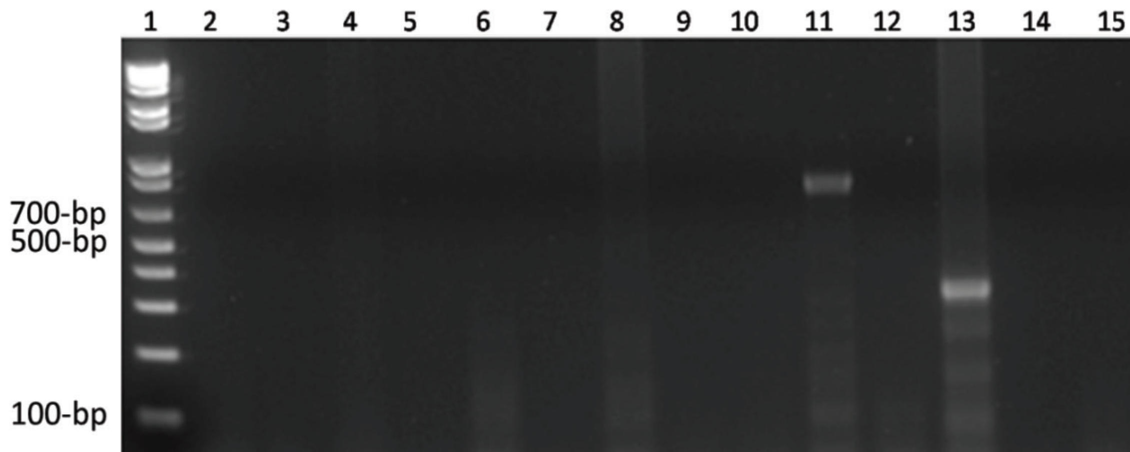

Figure 2. *Leptospira* spp. detection using nPCR. The nPCR amplification product for pathogenic *Leptospira* spp. has 730 bp, while non-pathogenic species can be observed with 400 bp only. Lane 1 is a 100-bp DNA Ladder. Lines 2–10 are negative samples, lane 11 is a positive sample for pathogenic *Leptospira* spp., and lane 13 is a positive sample for non-pathogenic *Leptospira* spp.

eradication program of *Leptospira* spp. infection should include detection of the microorganism itself among carrier and reservoir host animals.

Amplification of β-actin gene

The DNA templates extracted through boiling of urine were of good quality as indicated by bright, single band in all samples on agarose gel after electrophoresis.

Leptospira *spp. detection using LAMP*

Using LAMP, *Leptospira* spp. was detected in the boiled urine samples. The results of the amplification were interpreted after the addition of SYBR Green dye and agarose gel electrophoresis (Table 2). After the addition of SYBR Green dye, the results showed that six samples from water buffaloes, 10 samples from dogs, four samples from sewage rats, seven samples from pigs, one sample from goat, six samples from the cat, and two samples from cattle were observed positive for *Leptospira* spp.

After gel electrophoresis, nine samples from dogs and water buffaloes were positive for *Leptospira* spp. It was followed by pig (7), cat (6), sewage rats (4), cattle (2), and goat (1). These results were based on the presence of multiple ladder-like bands pattern after gel electrophoresis (Fig. 1).

Table 2. Comparison of LAMP results using agarose gel electrophoresis and color change using dye and nPCR results.

Animal (*n* = 20 each)	Dye	Gel electrophoresis
Buffalo	6	9
Dog	10	9
Rat	4	4
Pig	7	7
Goat	1	1
Cat	6	6
Cattle	2	2

Detection of **Leptospira** *spp. by nPCR*

In nPCR, urine samples observed to have 400 and 730 bp on agarose gel electrophoresis regardless of being non-pathogenic or pathogenic, respectively, were considered positive. The results showed that four samples from water buffalo, three samples from dogs, three samples from rats, two samples from pigs, and one sample from goats were positive for *Leptospira* spp. Out of 13 positive samples from *Leptospira* spp., only four demonstrated an amplicon size of 730 bp on agarose gel electrophoresis**.** Those samples came from dog (1) and water buffaloes (3). A sample coming from water buffalo, which was positive for the pathogenic strain of *Leptospira* spp. as observed on agarose gel electrophoresis was sent for sequencing. Sequence analysis demonstrated 98% homology to the known pathogenic strains of *Leptospira* spp. (data not shown).

Efficiency of LAMP as compared with nPCR in the detection of **Leptospira** *spp.*

The positivity rate in each animal commodity was determined in each method and was compared. Positivity rate is the measure of the proportion of cases which confirmed to be positive.

The samples were in replicates during the experiment and were repeated several times. Results showed that the positivity rate is higher in the LAMP assay as paralleled to the nPCR for each animal commodity. Only 13/140

(9.3%) of the animals were positive for *Leptospira* spp. using nPCR, while LAMP detected 40/140 (29%) samples were positive.

Discussion

Leptospira *spp. detection using LAMP*

The interpretation of LAMP result using dye and agarose gel electrophoresis varied in some samples. The urine samples from sewage rats, pigs, cats, goat, and cattle commodities were consistently positive using the two indicators, whereas the samples from dog and buffalo did not demonstrate consistency (Table 2). In a previous study, minimal errors of 3/188 (1.6%) and 1/229 (0.4%) inconsistency were obtained due to the differences in the interpretation of readers of LAMP result using dye [16]. The difference between the results of the dye test and agarose gel electrophoresis of the PCR products may be due to the subjectivity of the interpretation of the dye test result by the reader. In addition, Zhang et al. [17] reported that gel electrophoresis is more sensitive than color change by naked eye using SYBR green. Therefore, the result of LAMP assay after gel electrophoresis was used for the computation of positivity rate among animal commodities.

Japanese researchers developed LAMP [18] to overcome some of the drawbacks of PCR. Compared with PCR, the result of this molecular technique is faster and claimed to be more specific and sensitive. More than that, this technique is also considered as more cost-effective compared with PCR because LAMP does not need sophisticated laboratory equipment like thermal cycler. Heat block can be used for incubation of the samples. With this, LAMP was considered as more economical and cost-effective molecular test than PCR [3,14,16,19].

Detection of Leptospira *spp. by nPCR*

Several laboratory techniques were developed to aid in the rapid screening and early disease detection [2,20,21]. PCR, in particular, has revolutionized the disease detection methods in many areas or industries. This technique, being widely used became a routine diagnostic procedure in most of the reference laboratories.

Evaluation of the PCR products that involved in the use of Leptospira-nPCR primers (*flab* f1/r1 and *flab* f2/r2) targeted the *flab* gene of the *Leptospira* spp. Results showed that four of 13 positive samples or 30.76% demonstrated an amplicon size of 730 bp. One sample came from the dog and the three from the Buffalo group. Both parameters (dye color change and gel electrophoresis) had higher positive results than that of nPCR. Nevertheless, the results of gel electrophoresis of LAMP showed higher sensitivity compared with nPCR which concurred with the study of Koizumi et al. [14].

Koizumi et al. [22] stated that antibiotic therapy can decrease the detection capability of nPCR. Antibiotic therapy may be the reason for the lower positive results of nPCR as compared with LAMP. During the collection of samples of this study, most of the animals were given antibiotic therapy due to clinical signs and herd history to leptospirosis. In addition, antibiotic therapy may decrease the leptospiral cells in urine up to the level nPCR cannot detect.

Efficiency of LAMP as compared with nPCR in the detection of Leptospira *spp.*

The detection of *Leptospira* spp. using LAMP and PCR showed that LAMP has higher sensitivity and specificity, which could be identical to that of nPCR or real-time PCR [3]. In this study, positive samples as confirmed through nPCR were also positive to LAMP which was in agreement with the previous studies. In addition, two leptospiral cells per reaction mixture were demonstrated from the previous study utilizing the same primers [14]. This detection limit is lower from previously reported LAMP protocols with 100 leptospiral cell per reaction mixture [3] and 10 leptospiral cells per reaction mixture detection limit [23]. This low detection limit could indicate that LAMP protocol used in this study could have higher sensitivity as compared with the nPCR protocol (Table 3). According to the WHO, leptospirosis is possibly the most prevalent and predominant disease worldwide [2]. Domestic and wild animals are considered to be the carriers or host reservoirs of this bacterium [24], while most of the reported outbreaks are associated to the existence of wild rodents in the environment. In this study, it was observed that there were no positive samples coming from cats in both nested PCR and LAMP method. Previously, there is a notion that cats are resistant or resilient to leptospirosis infection or development of leptospirosis infection, [25] since the signs and symptoms of leptospirosis is difficult to recognize or is less frequent as compared with other animals [26]. However, serological studies revealed that cats are exposed to *Leptospira* spp., however, clinical signs and symptoms are rarely seen or reported [20,21]. Leptospiremia and leptospiruria had been reported

Table 3. Comparison of LAMP and nPCR results.

Animal (*n* = 20 each)	LAMP	nPCR
Buffalo	9	4
Dog	9	3
Rat	4	3
Pig	7	2
Goat	1	1
Cat	6	0
Cattle	2	0
Positivity rate	29.0%	9.3%

to be observed in experimentally infected cats nonetheless, the infection or clinical signs observed are generally mild. Reports also include the presence of renal and hepatic inflammation in cats after the experiment infection [27]. Cats, therefore, has an important part in the epidemiology of *Leptospira* spp. since they can be carriers and shedders of this organism without apparent clinical signs of infection. Serovars Canicola, Grypotyphosa, and Pomona were reported to have been successfully isolated from cats [27]. Cats, like dogs, were also reported to shed *Leptospira* spp. intermittently in the urine several weeks up to 3 months following the experimental infection [28]. These findings support that cats can excrete potential zoonotic leptospires, and can contaminate the environment [27]. In addition, several studies concluded that cats could possibly infect the environment or transmit the disease to people if and only if the environmental conditions are optimal [26,28,29].

Other domestic animals like dogs even those vaccinated against *Leptospira* spp. can shed organisms through urine and this may result with the transmission of the infectious microorganism to humans. With this, dogs can become a significant reservoir of *Leptospira* spp. that may infect humans [4,5]. However, rodents are the most frequent carriers of this bacterium [30]. In this study, the positive animals have probably been exposed into a *Leptospira* spp. contaminated environment and eventually developed an infection. In rural settings, it is expected that there is a higher risk of *Leptospira* spp. seropositivity due to the presence of livestock, small animals and rodents, which are the usual reservoirs of this bacterium [26]. Leptospirosis which is voided in the urine of an infected animal can live for weeks to months in water and soil. The organism can continue to infect the animals in the surroundings for months, especially in the areas with slow moving or stagnant water. Animals become infected through direct contact in the contaminated water or soil. It is known that the bacteria can enter abraded skin or mucous membranes. In some studies, it is reported that the animals may get infected with the bacteria through drinking of the *Leptospira* spp. contaminated water and through inhalation of contaminated air [2]. In other study, it was reported that the presence of rat in the environment can increase the possibility of harboring *Leptospira* spp. [22].

Conclusion

The LAMP is more sensitive, economical, and faster assay than PCR. Previous research studies cited that that LAMP is not only rapid and cost-effective but also an accurate established nucleic acid amplification method that had been developed for the detection of diverse pathogens and had been made commercially available. In addition, LAMP has a potential application as pen-side screening, surveillance, and clinical diagnostic kits of infectious diseases without requiring advance equipment and skilled personnel.

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Conflict of Interest

The authors have no conflict of interest to declare with respect to the research, authorship, and publication of this research article.

Authors' Contributions

Gabriel Alexis SP. Tubalinal and Michelle M. Balbin performed the research activity, compiled the data, and prepared the manuscript. Michelle M. Balbin, Marvin A. Villanueva, Clarissa Yvonne J. Domingo, and Claro N. Mingala designed the research experiment, analyzed the data, and prepared the manuscript. Claro N. Mingala for the final approval of the manuscript to be published. All authors have read and approved the manuscript before submission.

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