รายงานวิจัยฉบับสมบูรณ์

การพัฒนาวิธีการตรวจวินิจฉัยโรคเลปโตสไปโรซีส (โรคฉี่หนู)

โดยวิธี Loop-mediated isothermal amplification

โดย

ดร.เพียงจันทร์ สนธยานนท์

มีนาคม 2554

สัญญาเลขที่ MRG5280067

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(Rapid diagnosis of leptospirosis by loop-mediated isothermal amplification)

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Abstract

Project Code: MRG5280067

Project Title: Rapid diagnosis of leptospirosis by loop-mediated isothermal amplification

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Leptospirosis has the greatest impact on health in developing countries, but there is a paucity of diagnostic tests that are suitable for technology-restricted settings. We describe the development and clinical evaluation of loop-mediated isothermal amplification (LAMP) targeting the 16S ribosomal RNA gene (*rrs*) of *Leptospira* spp. belonging to the pathogenic and intermediate groups. The lower limit of detection was 20 genomic equivalents/reaction, and the analytical specificity was high with positive LAMP reactions for pathogenic and intermediate group *Leptospira* spp. but negative reactions for non-pathogenic *Leptospira* spp. and a range of other bacterial species that may cause acute febrile illness. A case-control study was conducted in northeast Thailand to evaluate assay performance in 133 patients with laboratory-proven leptospirosis based on culture and/or the microscopic agglutination test (MAT), and 133 patients with other causes of acute febrile illness. Using whole DNA extracted from EDTA blood taken at presentation, *rrs* LAMP was positive in 58/133 cases (sensitivity 43.6; 95%CI: 35.0-52.5), and 22/133 controls (specificity 83.5; 95%CI: 76.0-89.3). Diagnostic sensitivity was higher for a subset of 39 patients who were culture positive for *Leptospira* spp (84.6; 95%CI: 69.5-94.1). Our results indicate that *rrs* LAMP can provide a rapid result in around half of patients with leptospirosis at the time of presentation, but its diagnostic utility is reduced by an imperfect specificity.

Keywords: Leptospirosis; Loop-mediated isothermal amplification; diagnosis

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ชื่อโครงการ การพัฒนาวิธีการตรวจวินิจฉัยโรคเลปโตสไปโรซีส (โรคฉี่หนู) โดยวิธี Loop-mediated isothermal amplification

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ภาควิชาชีวโมเลกุลและพันธุศาสตร์โรคเขตร้อน คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

ระยะเวลาโครงการ มีนาคม 2552 ถึง มีนาคม 2554

โรคเลปโตสไปโรซีสหรือโรคไข้ฉี่หนูเป็นปัญหาสำคัญด้านการสาธารณสุขในหลายประเทศ เนื่องจากวิธีการตรวจวินิจฉัยโรคยังคงมีข้อจำกัดในด้านอุปกรณ์และงบประมาณที่จะนำไปใช้ในทุกพื้นที่ที่มีการแพร่ระบาด ผู้วิจัยได้ทำการพัฒนาวิธีการตรวจวินิจฉัยโรคเลปโตสไปโรซีส โดยวิธี Loop-mediated isothermal amplification (LAMP) จากยีน 16S rRNA ของเชื้อ *Leptospira* โดยสามารถตรวจสอบในห้องปฏิบัติการได้ต่ำสุดในระดับ 20 genomic equivalents/reaction และมีความจำเพาะต่อเชื้อ *Leptospira* สายพันธุ์ที่ก่อโรค (pathogenic and intermediate group) และไม่จำเพาะต่อเชื้อ *Leptospira* สายพันธุ์ที่ไม่ก่อโรค รวมทั้งเชื้อแบคทีเรียชนิดอื่นๆ ที่อาจพบในผู้ป่วยโรคไข้ไม่ทราบสาเหตุ นอกจากนี้ ผู้วิจัยได้ทำการประเมินผลวิธีการวินิจฉัยโรคเลปโตสไปโรซีสโดยวิธี LAMP ที่พัฒนาขึ้น แบบ case-control study โดยศึกษาในกลุ่มตัวอย่างจำนวน 266 ตัวอย่าง ได้แก่ กลุ่มผู้ป่วยโรคเลปโตสไปโรซีส จากการให้ผลบวกด้วยวิธีการเพาะเชื้อและ/หรือวิธีการทางอิมมูโนโลยี(Microscopic agglutination test) จำนวน 133 ราย และกลุ่มควบคุม 133 ราย โดยทำการตรวจสอบในตัวอย่างดีเอ็นเอที่สกัดได้จากเลือดของผู้ป่วยและกลุ่มควบคุม พบว่า วิธี LAMP ให้ผลบวกในกลุ่มผู้ป่วย 58 ใน 133 รายกล่าวคือมีความไวในการวินิจฉัย(sensitivity) เท่ากับร้อยละ 43.6 (95%CI: 35.0-52.5) และ มีความจำเพาะในการวินิจฉัย (specificity) ร้อยละ 83.5 (95%CI: 76.0-89.3). อย่างไรก็ตาม ความไวในการตรวจวินิจฉัย (Diagnostic sensitivity) เมื่อเปรียบเทียบกับวิธีการเพาะเลี้ยงเชื้อเพียงอย่างเดียวมีค่าสูงถึงร้อยละ 84.6 (95%CI: 69.5-94.1) กล่าวโดยสรุปคือวิธีการที่ได้พัฒนาขึ้นดังกล่าว แม้ว่าจะให้ผลตรวจที่รวดเร็วและแม่นยำมากในห้องปฏิบัติการ เมื่อนำไปใช้ในการตรวจตัวอย่างผู้ป่วยจากแหล่งระบาด พบว่าให้ผลบวกเพียงครึ่งหนึ่งของผู้ป่วยเท่านั้น โดยรายงานนี้จะได้นำเสนอในรายละเอียดต่อไป

คำหลัก: โรคเลปโตสไปโรซีส; Loop-mediated isothermal amplification; การตรวจวินิจฉัย

Introduction

Leptospirosis is an acute febrile disease caused by pathogenic *Leptospira* species. The disease is distributed worldwide but is most common in tropics[1]. The clinical features of disease are board ranging but similar to other bacteria which found co-exist in the endemic area such as scrub typhus, dengue and malaria[2]. Therefore, accurate laboratory diagnosis is essential for patient management. Culture is the gold standard diagnostic method during the acute phase but is rarely undertaken because of time consuming. The microscopic agglutination test (MAT) relies on antibody detection which are useful for serovar or serogroup identification but less specificity for individual cases of human leptospirosis[3]. Molecular-based method such as PCR, multiplex PCR and real-time PCR for pathogenic *Leptospira* detection have been reported [4, 5, 6, 7]. However, current laboratory tests available are not in routine use and provide rapid diagnosis answer since they are costly, difficult to perform and with the exception of PCR provide a retrospective diagnosis.

Loop-mediated isothermal amplification (LAMP) is an alternative method of rapid DNA amplification. The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand[8]. The reaction results in the accumulation of 10^9 copies of target and simply requires a laboratory water bath or heating block to maintain a constant temperature of 60-65°C, making it particularly suited to lower technology settings. LAMP has been developed for the detection of a range of pathogenic bacteria and viruses [9, 10, 11], including *Leptospira* spp[12]. The published leptospira assay targeted *lipL41*, a gene encoding the outer membrane protein LipL41 which has been shown to be expressed by pathogenic *Leptospira* spp. [13]. The lower limit of detection was reported to be around 100 copies and the assay was specific for *Leptospira* in a laboratory evaluation. The assay detected *Leptospira* in 7 mouse kidney samples taken from animals captured during environmental surveillance [12], although the assay has not undergone clinical evaluation to date.

Leptospira genomes contain two 16S rRNA genes for which there are multiple sequences of variable length in public databases[14], phylogenetic analysis of which has revealed three clades within the genus composed of the pathogenic species, nonpathogenic species, and a clade with intermediate 16S rRNA gene sequence relatedness. The latter group is considered to represent opportunistic/intermediate pathogens, although there are a gradually increasing number of reports associating them with human leptospirosis [15, 16, 17]. The objective of this study was to utilize the

phylogenetic distinctions inherent in the 16S rRNA gene sequence to develop an alternative LAMP assay that can detect *Leptospira* spp. in both the pathogenic and intermediate groups, and to undertake a clinical case-control evaluation of the diagnostic acccuracy of both the LipL41 and the 16S rRNA assays in rural Thailand.

Literature review

Taxonomy and Classification

Leptospira are obligate aerobic motile spirochetes with an the optimum growth temperature of 28-30 ºC[18]. *Leptospira* can enter the human body through cuts and abrasions in the skin and through mucous membranes of the eyes, nose and mouth. Human infection occurs following direct contact with the urine of infected animals or by contact with a urine-contaminated environment such as surface water, soil and plants. *Leptospira* have been isolated from a wide range of wild and domestic animals, including rodents, insectivores, dogs, cattle, pigs and horses. Leptospirosis is an occupational hazard for people who work outdoors or with animals, especially agricultural workers such as rice and sugar-cane field workers[19], farmers, sewer workers, veterinarians, dairy workers and military personnel. It is also a recreational hazard to those who swim in contaminated waters.

Leptospira belong to the genus *Leptospira*, family *Leptospiraceae*. Using DNA-DNA hybridization[20] and 16S rRNA sequencing[14], the genus *Leptospira* has been divided into 17 species. These species can be grouped into pathogenic, intermediate and saprophytic *Leptospira*. Pathogenic *Leptospira* include *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii*, *L. alexanderi*, *L. borgpetersenii*, *L. alstonii* (*L. genomospecies* 1), and *L. noguchii* while intermediate *include L. inadai, L. fainei, L. broomi*. Saprophytic *Leptospira* include *L. biflexa, L. wolbachii, L. meyeri, L. vanthielii (L. genomospecies 3), L. terpstrae (L. genomospecies 4) and L. yanagawae (L. genomospecies 5)*[14, 21]. *Leptospira* have also been classified into over 200 serovars based on the cross-agglutination absorption test (CAAT), which is based on agglutinating patterns of live organisms in the presence of a panel of test sera[3]. Serovars are grouped into serogroups on the basis of shared antigens and agglutinating patterns [22].

Clinical features and laboratory diagnosis

Clinical features vary and often mimic other acute febrile illnesses such as dengue, malaria and rickettsial infections. Leptospirosis has two phases of illness: acute or septicemia (leptospiraemia) and immunity (leptospiruria), as shown in Figure 1[18]. The acute phase can last for a week, followed by the immune phase which is characterized by antibody production. The

latter phase can last for an extended period (2-6 months)[22]. *Leptospira* are presented in blood and cerebral spinal fluid (CSF) during the acute phase and are excreted in the urine during the immune phase.

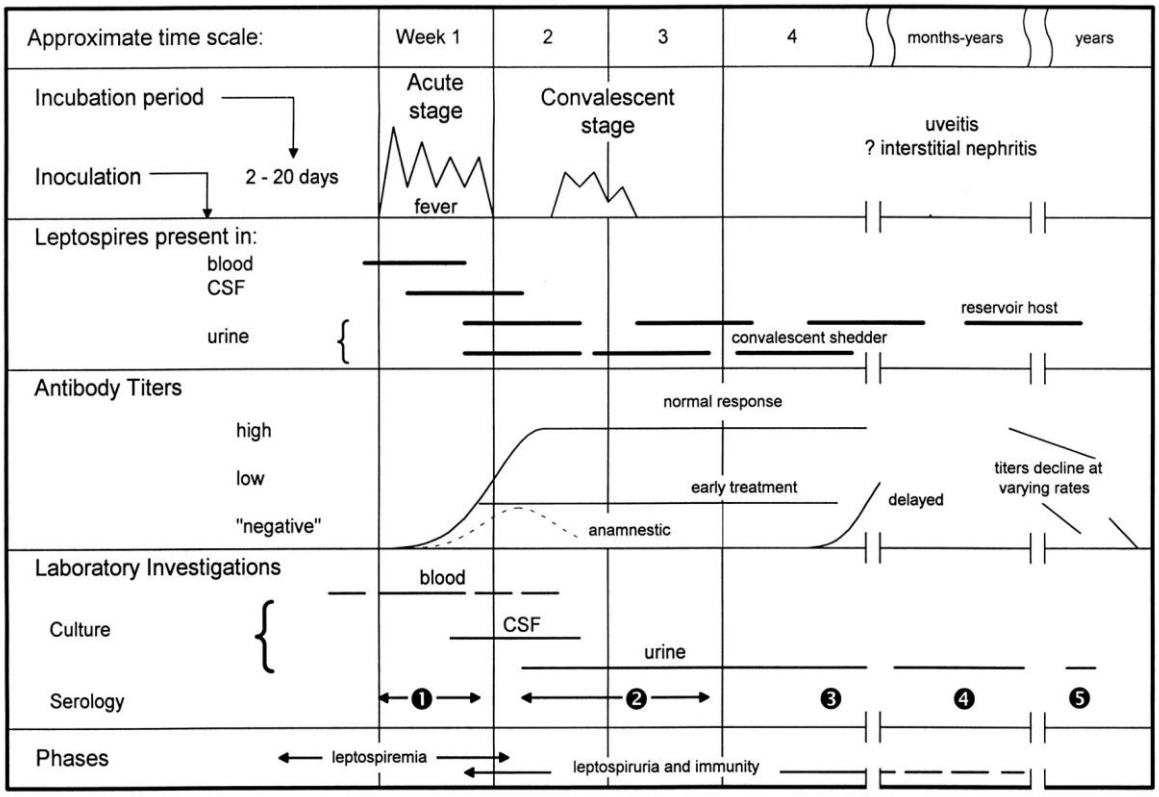


Figure 1. Phases of leptospirosis. Reproduced from Levett, *et al* [18]

Laboratory diagnosis of leptospirosis

1. Culture

It is a diagnostic gold standard method during the acute phase but is rarely undertaken because it is slow, time consuming and expensive. Primary culture requires 13 weeks or longer, during which cultures are examined each week[18]. Culture is performed using Ellinghausen, McCullough, Johnson and Harris (EMJH) medium. Isolation has been reported from a range of clinical sample types, including whole blood, surface and spun plasma, clotted blood, CSF, and fluid from the eye [23]. Culture has a low diagnostic sensitivity (40%), but is necessary in order to obtain a collection of strains for use in the CAAT assay.

2. Serological methods

Leptospira-specific enzyme-linked immunosorbent assay (ELISA) and lateral flow enzyme immunosorbent assay have been used during the acute phase but show low sensitivity and

specificity compared with the microscopic agglutination test (MAT) [24]. This is because antibodies are often absent early in disease, and MAT relies on paired sera taken during the acute and convalescent phases. MAT detects the presence of specific *Leptospira* antibodies using a panel of antigens in the form of live organisms that represent the serogroups or serovars present in the local area. MAT is labor intensive and only performed in a few references laboratory which routinely maintain live cultures of all serovars [18]. This is the reference serological method, but the highest titres obtain often fail to reflect the serovar of the infecting organisms in a given region [22]. The number of serological distinct strains of leptospires has increased over time.

3. Molecular diagnosis

Polymerase Chain Reaction (PCR) has been developed and used for the diagnosis of human leptospirosis. This method is useful for early diagnosis before antibodies are present[25]. Two PCR assays have been validated for use in clinical diagnosis [4, 25]. Multiplex PCR based on the 23S rRNA gene has been used to differentiate between pathogenic and saprophytic *Leptospira* spp.[7]. The ability to distinguish between pathogenic and saprophytic *Leptospira* is useful for epidemiological and public health purposes. Furthermore, real-time PCR for the detection of *Leptospira* using various gene targets including ribosomal RNA gene[5], major outer membrane lipoprotein (LipL32)[26], immunoglobulin-like protein (Lig)[6] and DNA gyrase gene (gyrB)[27] have also revealed high sensitivity for the diagnosis of leptospirosis. However, an important limitation of these methods in resource-restricted areas is the need for highly skilled staff and expensive equipment.

Loop mediated isothermal amplification (LAMP) was first described in the year 2000[8]. The method is based on auto-cycling strand displacement DNA synthesis performed by *Bst* DNA polymerase and a set of two specially designed inner and two outer primers that recognized a total of six distinct sequences on the target DNA. An extremely large amount of DNA is produced by the cycling reaction in less than an hour under isothermal conditions ranging from 60 to 65°C in a heat block or water bath. The final products are stem-loop DNA with several inverted repeats of the target that exhibit cauliflower-like structures with multiple loops.

In practice, LAMP is performed in a single closed tube at a single incubation temperature, thereby obviating the need for expensive thermal cyclers. The amplification product can easily be detected by the appearance of turbidity caused by white precipitation of magnesium pyrophosphate in solution[28], or with the additional of SYBR green[11], or cationic polymers to form an insoluble complex with DNA[29] which can be easily detected by the naked eye or using a conventional UV illuminator. Recently, the visual detection of product has been described after

addition of manganous ion and calcein (a fluorescent metal indicatior) to the reaction mix [30]. LAMP has been developed to detect many pathogenic bacteria and viruses [10, 11, 31, 32]. Evaluation of the method has shown it to have potential as a simple screening assay in the field or as a point-of-care test [33]. The method has high sensitivity and specificity for clinical diagnosis [34]. It can be applied to a range of clinical samples including plasma, serum, phosphate-buffered saline (PBS), saline, urine, aqueous humor and vitreous [35].

Recently, there was a report on "Application of a loop-mediated isothermal amplification method for the detection of pathogenic *Leptospira*" by X. Lin, Y. Chen, Y. Lu, J. Yan, J. Yan. in *Diagnostic Microbiology and Infectious Disease* 2009. 63 (3); pp. 237-242. The work was used loop-mediated isothermal amplification (LAMP) method target LipL41 gene as model. Despite the same LAMP technology, the article used a different target gene and the clinical validation of the assays has not been reported. Therefore, our study have validated both methods (*rrs*-LAMP and *LipL41*-LAMP) using the same sample set.

Materials and Methods

1. Gene selection and primer design

A total of 39 sequences for the full-length 16S rRNA gene of *Leptospira* spp. were downloaded from GenBank, of which 23 sequences were from 8 pathogenic *Leptospira* spp., 6 sequences were from 3 intermediate group *Leptospira* spp., and 10 sequences were from 6 non-pathogenic *Leptospira* spp. Accession numbers are as follows: Pathogenic species *Leptospira interrogans* (n = 10, accession no. AY631894, AY996790-4, AY996796-8, AY99680), *L. borgpetersenii* (n = 2, accession no. AY631884, 887899), *L. kirschneri* (n = 3, accession no. AY631895, AY996801-2), *L. alexanderi* (n = 3, accession no. AY631880, AY996803-4), *L. santarosai* (n = 2, accession no. AY631883, AY996805), *L. noguchii* (n = 1, accession no. AY631886), *L. alstonii* (n = 1, accession no. AY631881), *L. weilii* (n = 1, accession no. AY631877); Intermediate species *L. inadai* (n = 3, accession no. AY631887, AY631891 & AY631896), *L. broomii* (n = 1, accession no. AY796065), *L. fainei* (n = 2, accession no. AY631885, 996789); Non-pathogenic species *L. biflexa* (n = 2, accession no. AY631876, AY631893), *L. meyeri* (n = 3, accession no. AY631878, 631889, 631892), *L. wolbachii* (n = 2, accession no. AY631879, AY631890), *L. vanthielii* (n = 1, accession no. AY631897), *L. terpstrae* (n = 1, accession no. AY631888) and *L. yanagawae* (n = 1, accession no. AY631882). The partial 16S rRNA sequence of 3 recently described *Leptospira* spp. were also included (pathogenic *L. kmetyi* (n=1, accession no. AB279549), and intermediate group *L. licerasiae* (n = 9, accession no.

EF612280-8), and *L. wolffii* (n = 1, accession no. EF025496)). All sequences were aligned and compared using Clustal X version 1.83[36] and Genedoc (available from http://www.nrbsc.org/gfx/genedoc/index.html).[37] A set of five primers consisting of two outer primers (F3 and B3), two inner primers (forward inner primer (FIP), and backward inner primer (BIP)) and one loop primer (loop B (LBP)) were designed using PrimerExplorer version 4 (http://primerexplorer.jp/e/). The target sequences were regions that were specific to the 16S rRNA gene sequence of all pathogenic and intermediate but not non-pathogenic *Leptospira* spp., as shown in Figure 1. The LAMP primers are given as demonstrated in Table 1.

Table 1 List of LAMP primer sequence used in this study.

Gene	Primer	Sequence 5'-3'
16S RNA (rrs)	F3	CTTCGGATTGTAAAGTTCADT
	B3	CCAGACWYRWAGTTTCAARTG
	FIP (F1c-F2)	CACGTAGTTAGCCGGTGCTT-AGGGAAAAATAAGCAGYRATG
	BIP (B1-B2c)	CGTATGGTGCAAGCGTTGTTC-VCAGKTTTCACAYCTGACT
	LPB	GCGTAGGCGGAYWTGTA

2. The *rrs*-LAMP reaction assays.

LAMP targeting 16S rRNA gene (*rrs*-LAMP) was used for diagnosis of *Leptospira* spp. Firstly, the reactions were performed using the LoopAmp DNA amplification kit from EIKEN Chemical (Japan). In brief, a total reaction volume of 25 µl contained 0.2 µM each outer primer (F3, B3), 1.6 µM of each inner primer (FIP, BIP), 1x reaction mix (RM), 1 µl of *Bst* DNA polymerase and 5µl of genomic DNA of *Leptospira* spp. The reaction mixture was incubated at 63 ºC for 2 hours and heated at 80 ºC 5 min. The controls were genomic DNA of *L. interrogans* (pathogenic), *L. inadai* (intermediate) and *L. biflexa* (non-pathogenic), and the negative control was extracted human DNA.

3. The optimization of Loop-mediated isothermal amplification

In order to find the optimum condition for *rrs*-LAMP assays to get the lowest detection limit and high specificity, the parameters involved in amplification were optimized such as primer ratio and concentration; reaction temperature; incubation time etc.

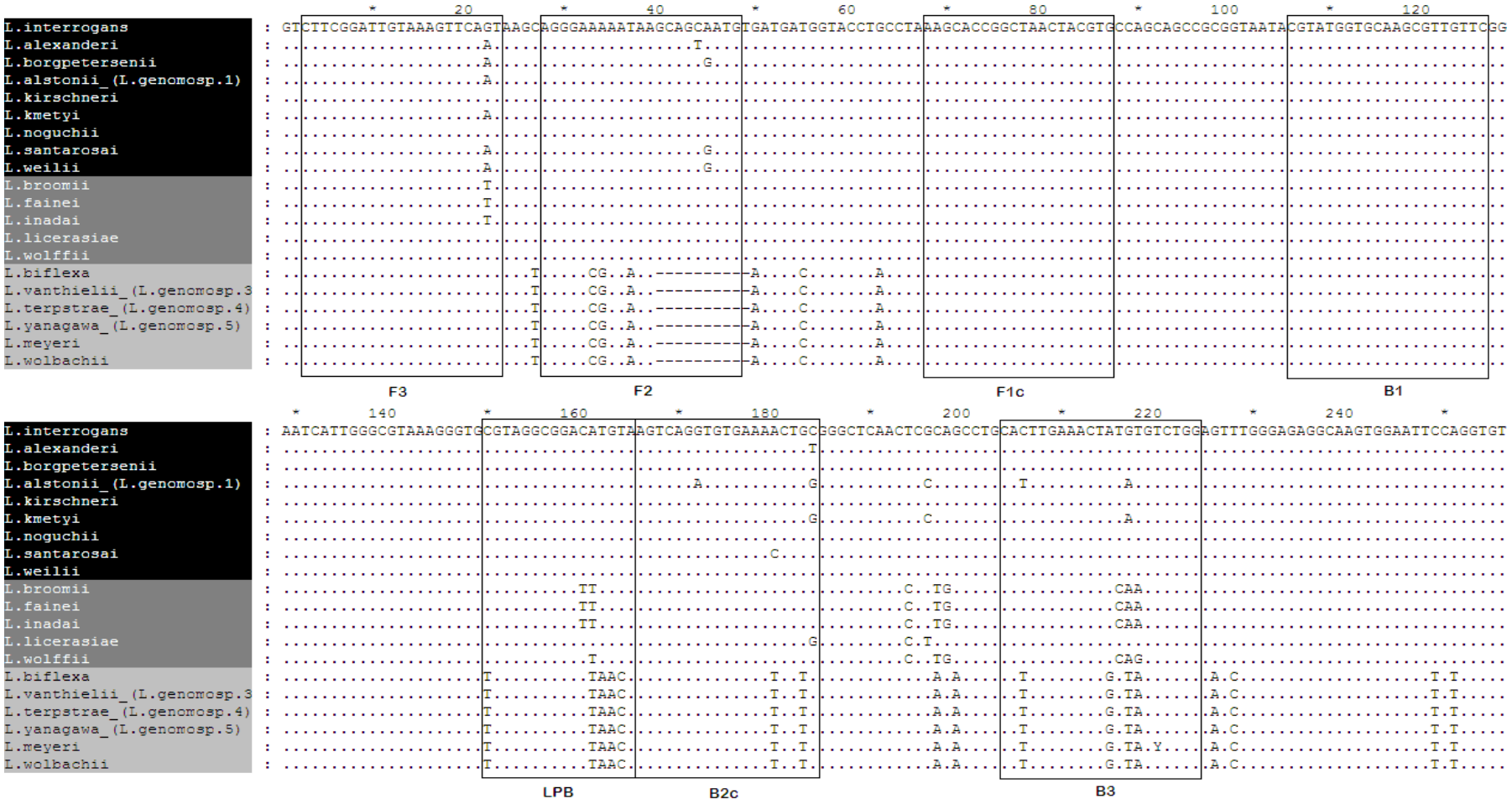


Figure 1 Sequence alignment of a region of the 16S rRNA gene of 20 *Leptospira* spp. to demonstrate the regions selected for primer design.

The pathogenic group represent by white letters in black box; intermediate group (white letters in grey box) and the non-pathogenic group (black letters in grey box). F1c, F2, F3, B1, B2c, B3 and LPB refer to the names and locations of target sequences for the primers used.

4. The specificity and detection limit of the assays

All *Leptospira* spp. used as reference strains in this study were listed in Table 2. Other bacteria including *Rickettsia* spp. (Rickettsiosis), *Orientia tsutsugamushi* strain Kato (scrub typhus), *Aeromonas spp. Staphylococcus aureus, Enterococcus spp., Burkholderia pseudomallei, Escherichia coli, Klebsiella pneumonia, Pseudomanas aeruginosa, Salmonella typhi,* and *Rickettsia typhi* that may be found in clinical samples were determined the specificity of assays. Serial dilutions of spiked bacterial DNA in whole blood taken from a healthy donor were performed to determine the detection limit of the method in blood.

5. Clinical validation

Patients with (cases) or without leptospirosis (controls) were selected from a prospective cohort study of acute febrile illness conducted at the Udon Thani Regional hospital, northeast Thailand between October 2000 and December 2001 [23]. In brief, patients were recruited into the study during twice daily ward rounds. Inclusion criteria were patients who were $\geq$15 years of age with fever (>37.8°C) of unknown cause who agreed to participate and to attend out-patient follow up for a convalescent serum sample. Exclusion criteria were patients with a blood smear positive for malaria parasites or those with other definable infections such as pneumonia or urinary tract infection. Blood was taken on admission from all patients for *Leptospira* culture, serological testing and a second (convalescent) sample was taken for serological testing around 2 weeks later.

Blood was collected for *Leptospira* culture (10 ml) into a sterile tube containing 250 units of heparin sodium (Heparin Leo®, Leo Pharma, UK). Culture was performed using 3 ml of EMJH supplemented with 3% rabbit serum and 0.1% agarose, as described previously [23]. Positive cultures were sent to the WHO/FAO/OIE Collaborating Center for Reference & Research on Leptospirosis, Australia for serovar identification using the cross agglutinin absorption test (CAAT) [38]. The microscopic agglutination test (MAT) was performed by the WHO/FAO/OIE Collaborating Center for Reference & Research on Leptospirosis, Australia as previously described [38], using a live panel of antigens representing both ubiquitous and locally prevalent serovars. A diagnosis of leptospirosis was based on isolation of *Leptospira* from blood and/or a positive MAT, which was defined as a 4-fold rise in titer between acute and

convalescence samples or a single titer of $\geq$ 1:400 or more. Patients who did not meet these criteria were defined as not having leptospirosis.

DNA was extracted from a 5 ml EDTA blood sample taken from cases and controls on admission and stored at -80°C prior to use. Extraction was performed using the Nucleon BACC 3 kit (Amersham), and the extract was suspended in 1ml TE buffer. 5 µl of extracted DNA were used in reaction. The assay was carried out in a reaction mixture of 25 µl containing 5 pmol of each outer primer (F3, B3), 40 pmol of each inner primer (FIP, BIP), 0.8 M Betaine (Sigma), 1.4 mM dNTP, 6 mM $MgSO_4$, 8 units of *Bst* DNA polymerase, Large Fragment (New England Biolab), 1x ThermoPol reaction buffer, and 5µl of DNA extracted from sample. The positive control used the low detection limit of the assays (1pg or equivalent to 20 copies per reaction) of laboratory cultures of *Leptospira* spp. The reaction mixture was incubated at 63 °C for 2 hours and then heated at 80 °C 5 min to terminate the reaction. The results were recorded by visualization of white precipitation after centrifugation and confirmed by 2% agarose gel electrophoresis.

5. Data analysis

All DNA samples were blindly tested and evaluated in duplicate for the assays using two LAMP assays (*rrs*-LAMP assays and *LipL41*-LAMPassays). A positive result for either one or both samples was interpreted as positive. The results were recorded and compared with the gold standard method (bacterial culture and/or MAT). Statistical analyses were performed using STATA/SE version 10.0 (College Station, Texas, United States). Diagnostic sensitivity (DSe) and specificity (DSp) of each PCR assay was defined against the combined result for culture and MAT (a positive result for either or both being interpreted as diagnostic for leptospirosis), and expressed as a proportion with exact 95% confidence intervals (CI). Comparison of DSe and DSp for the two LAMP assays was performed using the McNemar test. DSe of each assay was re-evaluated in the sub-set of patients who were culture positive.

Table 2. ***Leptospira*** **spp. used in this study**

Species	Serogroup	Serovar	Strain
L. interrogans	Autumnalis	Autumnalis	L0551
L. interrogans	Autumnalis	Autumnalis	Akiyami A
L. interrogans	Bataviae	Bataviae	Swart
L. interrogans	Djasiman	Djasiman	Djasiman
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. interrogans	Icterohaemorrhagiae	Lai	Lai
L. kirshneri	Grippotyphosa	Grippotyphosa	Moskva
L. kirschneri	Cynopteri	Cynopteri	3522C
L. borgpetersenii	Ballum	Ballum	Mus 127
L. borgpetersenii	Javanica	Javanica	Veldrat Batavia 46
L. santarosai	Autumnalis	Alice	Alice
L. alexanderi	Manhao	Manhao3	L60
L. noguchii	Autumnalis	Fortbragg	Fort Bragg
L. weilii	Celledoni	Celledoni	Celledoni
L. weilli	Sarmin	Sarmin	Sarmin
L. alstonii	Ranarum	Pingchang	80-412
L. inadai	Lyme	Lyme	10
L. biflexa	Semaranga	Patoc I	Patoc I
L. wolffii	Khorat	Khorat	H2
L .meyeri	Ranarum	Ranarum	Iowa City Frog
L. meyeri	Semaranga	Semaranga	Veldrat Semaranga 173
L. wolbachii	Codice	Codice	CDC
L. terpstrae	Icterohaemorrhagiae	Hualin	LT11-33
L. yanagawae	Semaranga	Saopaulo	Sao Paulo

Note: Isolates were obtained from the WHO/FOA/OIE/ Collaborating Center for Reference and Research on Leptospirosis, Australia; Bureau of Emerging Infection Disease, Ministry of Public Health, Thailand; American Type Culture Collection, USA; Royal Tropical Institute (KIT), Netherland; and Dr Thareerat Kalambaheti, Mahidol University, Thailand

Results

LAMP targeting 16S rRNA gene (*rrs*-LAMP) assays demonstrated initially the amplification on genomic DNA of 10 ng *L. interrogans* (pathogenic), 34 ng *L. inadai* (intermediate) but not to 10 ng *L. biflexa* (non-pathogenic) DNA and 500 ng human DNA as shown in Figure 2. Although, the assay did not amplify *Orientia tsutsugamushi* which caused scrub typhus and have been reported to be coinfected with leptospires in patients, the detection limit of assays was only 10 ng DNA of *L. interrogans* and 34 ng DNA of *L. inadai* as shown in Figure 3. We therefore further optimized the assays in order to get the lowest of detection limit which will increase the sensitivity of assays.

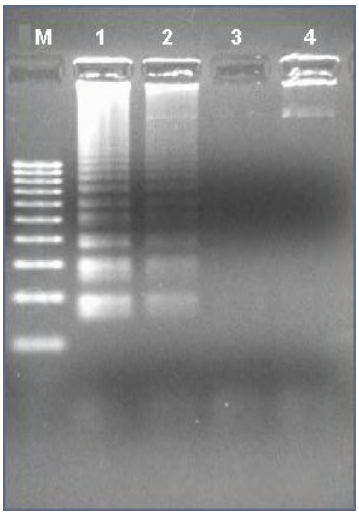


Figure 2. The amplification result of the *rrs*-LAMP assays on DNA of *L. interrogans* (1), *L. inadai* (2) and *L. biflexa* (3), and human DNA as negative control (4). Lane M is 100 bp DNA marker.

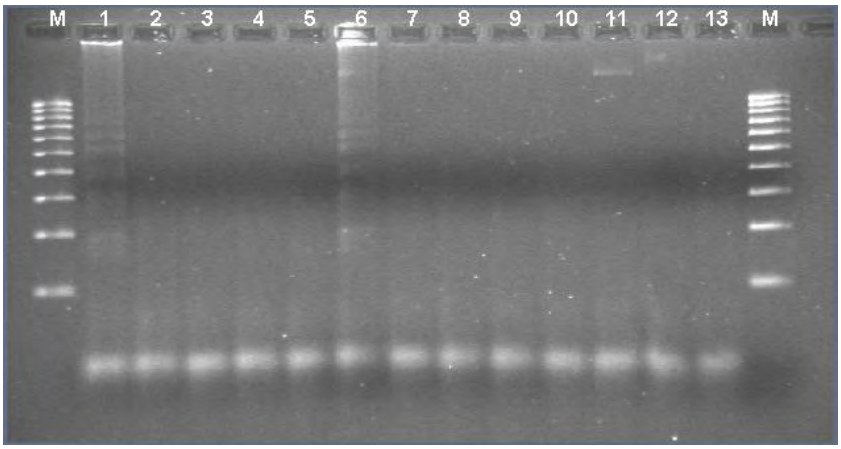


Figure 3 The *rrs*-LAMP assays on *L. interrogans* DNA dilution from 10 ng downward to 0.001 (lane1-5) and *L. inadai* DNA dilution from 34 ng to 0.003 ng (lane 6-10). Lane 11 was 10 ng *L. biflexa* DNA. Lane12 was 22 ng of *Orientia tsutsugamushi* DNA. Lane 13: no template control

Optimization of *rrs*-LAMP assays

Incubation temperature

The reaction temperature was incubated at various temperatures (63, 65 and 68 ºC) at 1 hour. The amplification product (as ladder pattern) was seen only at 63 ºC (Figure 4) while no product was shown at 65 and 68 ºC (data not shown). The detection limit for the assays was 1 ng DNA.

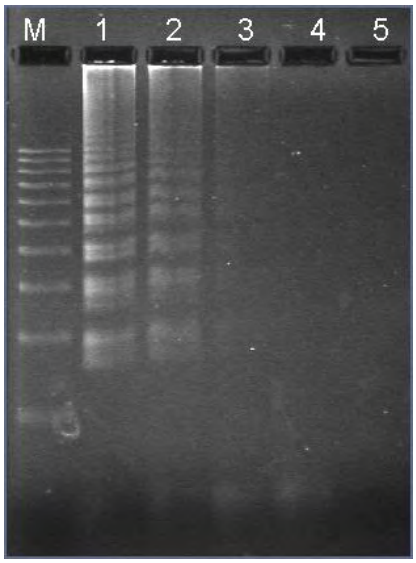


Figure 4 The *rrs*-LAMP assays incubated at 63 ºC for 1 hour using 10, 1, 0.1, 0.01, 0.001 ng of *L. interrogans* DNA

Use home-made reaction mixture

In order to drive down the assay costs, we used a home-made reaction mixture which has demonstrated the same level of detection limited. The home-made reaction mixture was mimicked the concentration which has been reported by the company. In brief, the assay was carried out in a reaction mixture of 25 µl containing 5 pmol of each outer primer (F3, B3), 40 pmol of each inner primer (FIP, BIP), 0.8 M Betaine (Sigma), 1.4 mM dNTP, 6 mM $MgSO_4$, 8 units of *Bst* DNA polymerase, Large Fragment (New England Biolab), 1x ThermoPol reaction buffer, and 1µl (approximately 20 ng-100 ng) of genomic DNA extracted from laboratory cultures of *Leptospira* spp. The reaction mixture was incubated at 63 ºC for 2 hours and then heated at 80 ºC 5 min to terminate the reaction. The results were visualized by centrifugation for 2 min at 13,000 rpm. The LAMP product was also assays by agarose gel electrophoresis. The result revealed the detection limit at 0.1 ng.

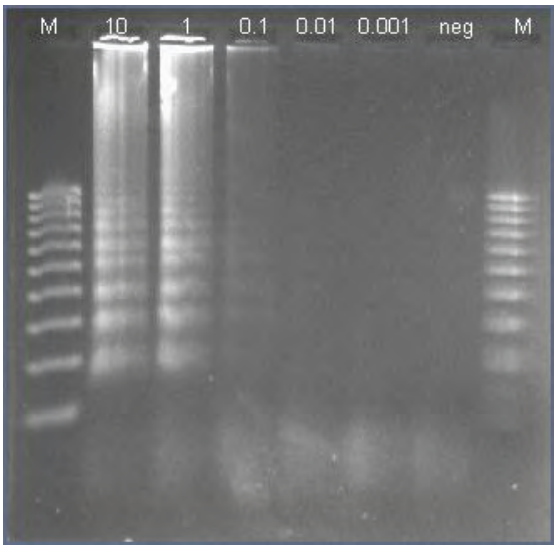


Figure 5 The *rrs*-LAMP assays using home-made reaction mixture. Genomic DNA of *L. interrogans* was varied from 10, 1, 0.1, 0.01, and 0.001 ng. neg: no template control (water):

Primer concentration

In order to reduce excess primer-dimers, primer F3/B3 were varied from 0.2 µM to be 0.15, 0.125, 0.1 and 0.05 µM while BIP/FIP were varied from 1.6 µM to be 1.2, 1, 0.8 and 0.4 µM. The results in Figure 6 showed that the optimum primer concentration of F3/B3 0.2 µM and those of FIP/BIP 1.6 µM

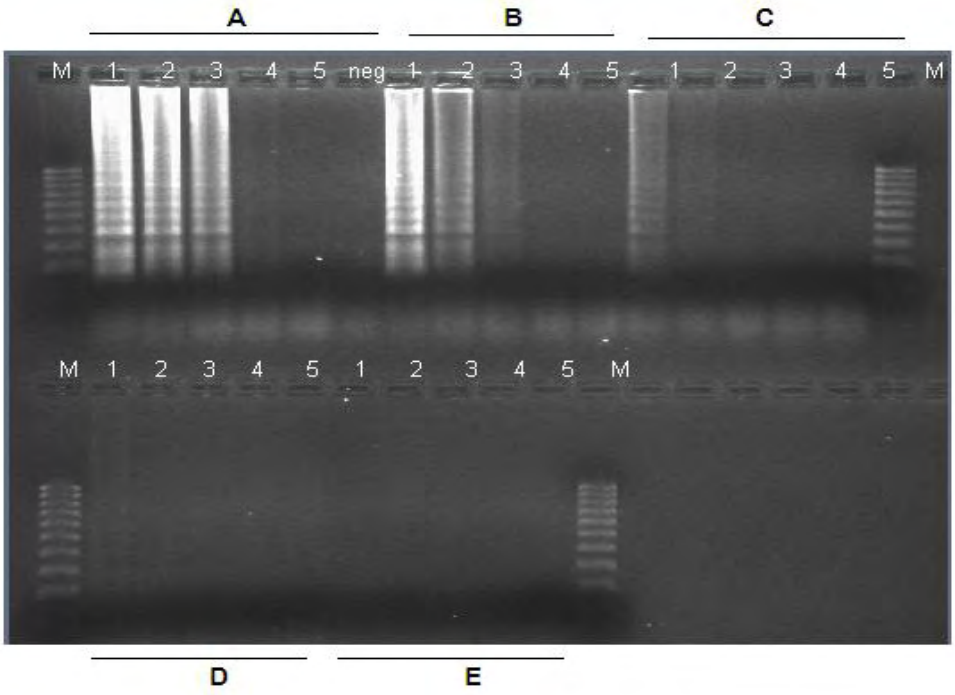


Figure 6. The *rrs*-LAMP assays using various primer concentrations as follows:
Panel A: F3/B3: FIP/BIP = 0.2: 1.6 µM; Panel B: F3/B3 : FIP/BIP = 0.15:1.2 µM;
Panel C: F3/B3: FIP/BIP = 0.125: 1.0 µM; Panel D: F3/B3: FIP/BIP = 0.1: 0.8 µM
Panel E: F3/B3: FIP/BIP = 0.05: 0.4 µM; Lane 1-5: *L. interrogans* DNA concentration 10, 1, 0.1, 0.01, 0.001 ng; neg: negative control (water) and M: 100bp DNA ladder

Primer ratio variation

In generally, the ratio of longer primer (FIP, BIP) to shorter primer (F3, B3) is 8:1 in the LAMP reaction. We have varied the ratio of primer from 8:1 to 7:1, and 6:1. The result showed that the optimal ratios were 8:1 and 7:1, as shown in Figure 7.

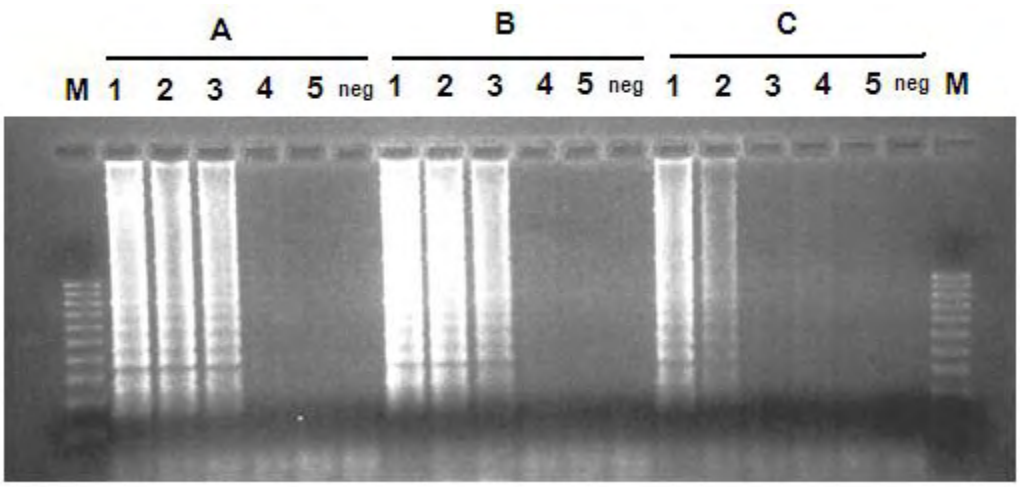


Figure 7. The *rrs*-LAMP assays on 0.1, 0.01, 0.001 ng of *L. interrogans* DNA (lane 1-5 respectively) using various primer ratios. Primer ratio (FIP/BIP: F3/B3) was 8:1(A), 7:1(B) and 6:1(C). Lane M: 100bp DNA ladder and neg: no template control (water).

Additional of a new loop primer.

There was a report on using a loop primer to accelerate the time for assay and increase the product[39]. A new loop primer (LPB) was designed and the position of was shown in Figure 1. The assay was carried out in a reaction mixture of 25 µl containing 5 pmol of each outer primer (F3, B3), 40 pmol of each inner primer (FIP, BIP), 20 pmol of loop primer (LPB), 1x ThermoPol reaction buffer, 0.8 M Betaine (Sigma), 1.4 mM dNTP, 6 mM $MgSO_4$, 8 units of *Bst* DNA polymerase, Large Fragment (New England Biolab), and 1µl (approximately 20 ng-100 ng) of genomic DNA extracted from laboratory cultures of *Leptospira* spp. The result showed that new loop primer could increase the sensitivity of detection. The detection limit was 1 pg DNA (or 100 copies of gene target) as shown Figure 8.

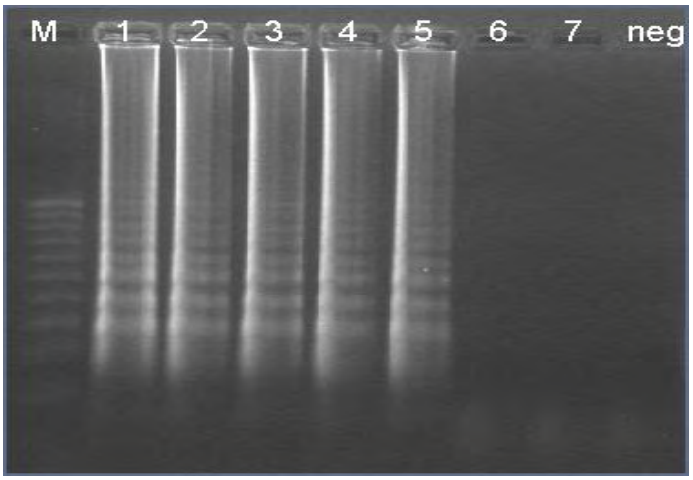


Figure 8 The *rrs*-LAMP assays with new loop primer (LPB) on *L. interrogans* DNA serial dilution from 10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg, 0.01 pg or equivalent to 10^6 $10^5$$10^4$ 10^3,10^2, 10, 1 copy of gene target respectively (lane 1-7). Lane M: 100bp DNA ladder; neg: no template control (water).

Incubation time

To reduce the incubation time, we have compared the incubation at 63 ºC for 1 hour and 2 hours. We preformed the assays by using total DNA extracted from EDTA blood from healthy volunteers and spiked with live *L. interrogans* serovar Autumnalis. Spiked whole blood samples were prepared as follows. A 5ml EDTA sample was drawn from a healthy volunteer. The bacterial count present in an aliquot of broth culture of *L. interrogans* serovar Autumnalis strain L0551 was quantified using a Petroff-Hausser counting chamber under dark-field microscope, as previously described [40]. Ten-fold serial dilutions (10^{-1} to 10^{-6}) of the starting culture were made, and 5 µl of each dilution added to separate aliquots of 200 µl EDTA blood. These were mixed and then immediately extracted using the QIAamp blood kit (Qiagen, Germany). 16S rRNA LAMP assay was determined using *L. interrogans* in 200 µl blood which equal to 5,000 , 500, 50, 5, 0.5, $5x10^{-2}$, $5x10^{-3}$ and $5x10^{-4}$ cell. The results showed that *rrs*-LAMP could detect 50 leptospira in a spiked cell in reaction (equivalent to 0.25 pg of DNA) by using loop primer with incubation time 2 hours, as shown in Figure 9.

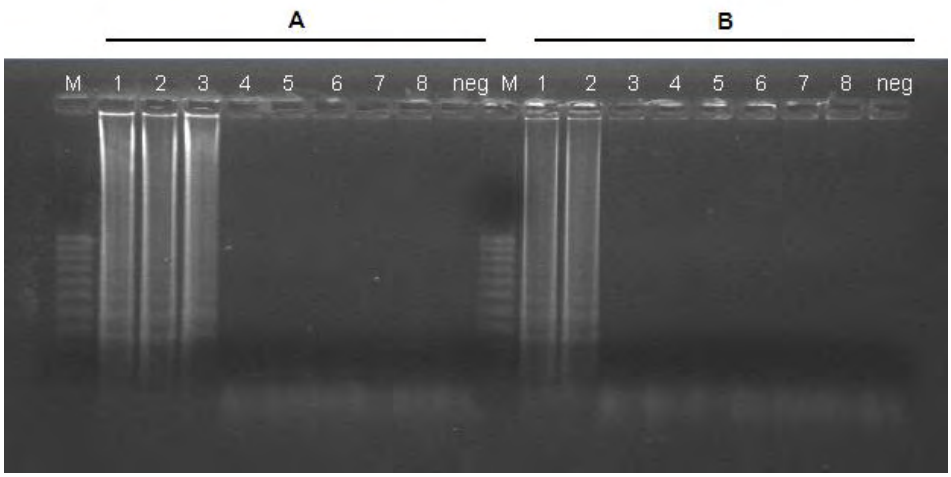


Figure 9. The *rrs*-LAMP assays on spiked *L. interrogans* cell in 200 µl blood which equal to 5,000 , 500, 50, 5, 0.5, $5x10^{-2}$, $5x10^{-3}$ and $5x10^{-4}$ cell (lane 1-8) at 63 ºC 2 hours (A) and 63 ºC 1 hour (B). Lane M: 100 bp DNA ladder; neg: no template control (water).

The specificity of assays

The *rrs*-LAMP assays were performed using genomic DNA of many bacteria such as *Aeromonas spp. Staphylococcus aureus, Enterococcus spp., Burkholderia pseudomallei, Escherichia coli, Klebsiella pneumonia, Pseudomanas aeruginosa, Salmonella typhi, Orientia tsutsugamushi* strain Kato and *Rickettsia typhi* in order to test specificity of the method.

Genomic DNA was extracted from all isolates using Wizard® Genomic DNA extraction kit (Promega, USA), with the addition of 60 µl of 10 mg/ml lysostaphin for the extraction of *S. aureus.* 5 µl (approximately 50 ng genomic DNA) were used in *rrs*-LAMP assays. The results showed that none of bacteria could be amplified except for *L. interrogans*. (Figure10).

The *rrs*-LAMP assays were performed using genomic DNA of *Leptospira* spp. including pathogenic, intermediate and non pathogenic strains. The results showed highly specific to pathogenic leptospires but not non-pathogenic and other related species (*Turneriella parva*) as shown in Figure 11.

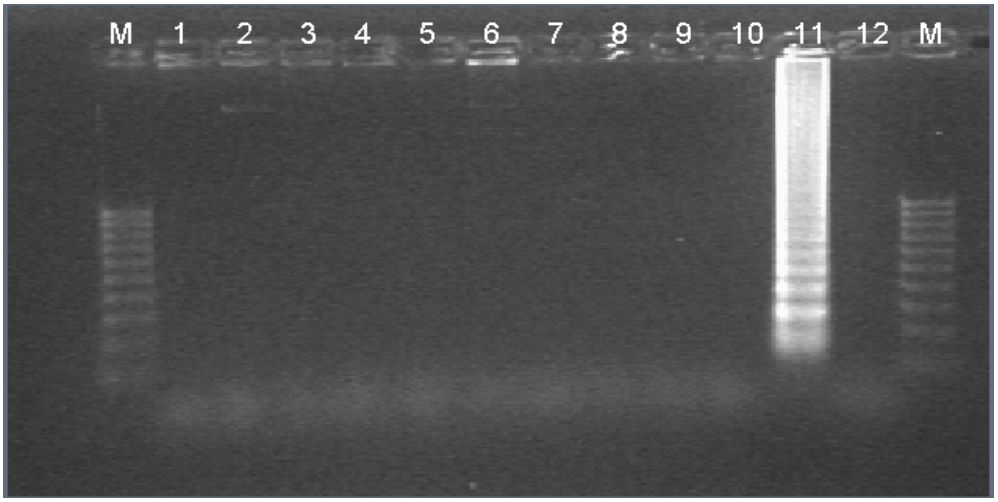


Figure 10 The *rrs*-LAMP assays on genomic DNA of various bacteria: Lane 1-10: *Aeromonas spp., B. pseudomallei, E. coli, Enterococcus* spp., *K. pneumonia, P. aeruginosa, S. typhi , S. aureus, O. tsutsugamushi strain Kato* and *R. typhi*. Lane 11: *L. interrogans.* Lane 12: no template control (water).

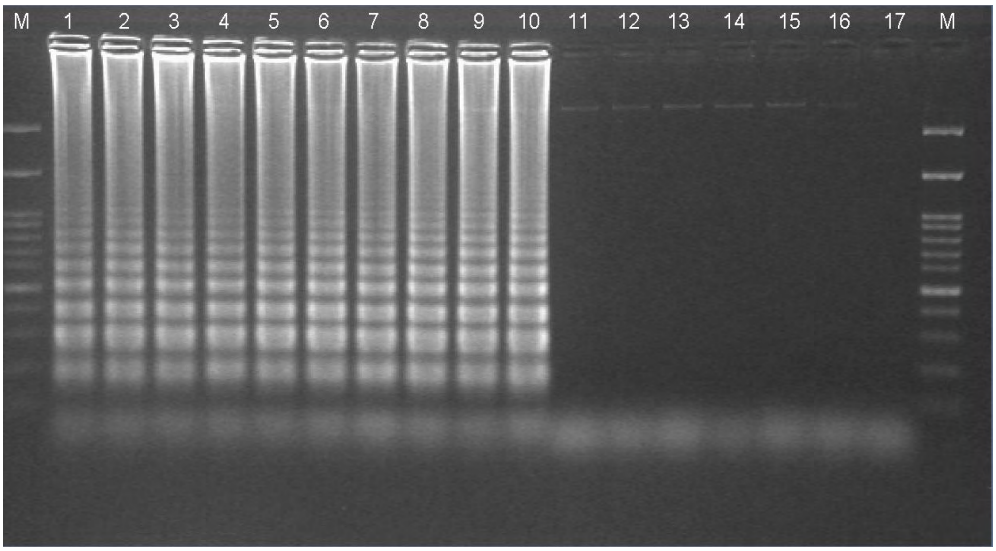


Figure 11 The *rrs*-LAMP assays on genomic DNA of *Leptospira* spp. Lanes 1-8, pathogenic *Leptospira* spp. (*L. interrogans*, *L. kirshneri*, *L. noguchii*, *L. alexanderi*, *L. weilli*, *L. borgpetersenii*, *L. santarosai, L. alstonii*, respectively). Lanes 9-10, intermediate group *Leptospira* spp. (*L. inadai* and *L. wolffii,* respectively). Non-pathogenic species (Lanes 11-16: *L. biflexa*, *L. meyeri*, *L. wolbachii*, *L. terpstrae*, *L. yanagawae* and *Turneriella parva*, respectively). Lane M, 100 bp DNA marker. Lane 17, negative (no template) control.

The detection limit of *rrs*-LAMP assays.

To determine the detection limit of the assay, we performed *rrs*-LAMP assays on serial dilution of genomic DNA extracted from pure laboratory culture of *L. interrogans* serovar Autumnalis. The dilution started from 10 ng downward to 0.01 fg per reaction or equivalent to $2x10^7$, $2x\ 10^6$, $2x10^5$, $2x10^4$, 2,000, 200 , 20, 2 , 0.2 0.02 and 0.002 copy of gene target per reaction respectively. The detection limit of the assays was 0.1 pg (or equivalent to 20 copies of gene target per reaction) as shown in Figure 12. Besides, the assays could amplified intermediate leptospires (*L. inadai*) while did not amplify non-pathogenic leptospire (*L. biflexa*).

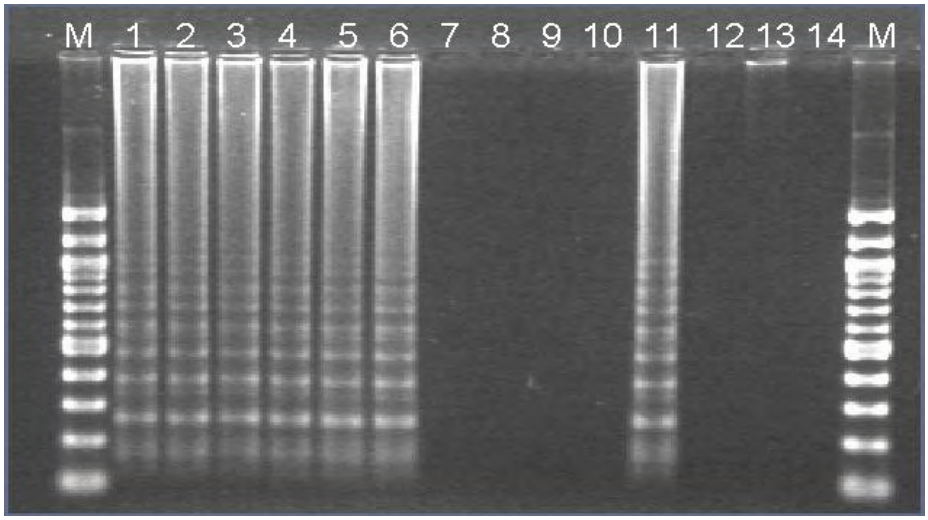


Figure 12 The *rrs*-LAMP assays on serial dilution of genomic DNA extracted from pure laboratory culture of *L. interrogans* serial dilution from 10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg, 10 fg, 1 fg, 0.1 fg 0.01 fg (lane 1-10). Lane 11: *L. inadai* DNA. Lane 12: *L. biflexa* DNA. Lane 13 : human DNA. *Lane* 14: no template control (water)

LAMP targeting Lipl41 reaction assays.

LAMP targeting *lipL41*gene (*lipL41*-LAMP) was used for diagnosis of *Leptospira* spp. by followed the protocol previously described [12]. In brief, a total reaction volume of 25 µl contained 5 pmol each outer primer (F3, B3), 40 pmol of each inner primer (FIP, BIP), 20 pmol of Loop primer (LPB), 1x reaction mix (RM), 1 µl of *Bst* DNA polymerase and 1µl of genomic DNA of *L. interrogans*. The reaction mixture was incubated at 63°C for 1 hour and heated at 80°C 5 min. The positive controls were serial dilution of *L. interrogans* genomic DNA from 10^5

to 1 copy/µl reaction, genomic DNA of *L. inadai* (intermediate), *L. biflexa* (non-pathogenic), and *Orientia tsutsugamushi* DNA. The results showed that primers were specific to 100 copies/µl reaction of *L. interrogans* (pathogenic) but not to 34 ng *L. inadai* (intermediate) and 10 ng *L. biflexa* (non-pathogenic) DNA and 30 ng *O. tsutsugamushi* DNA as shown in Figure 13.

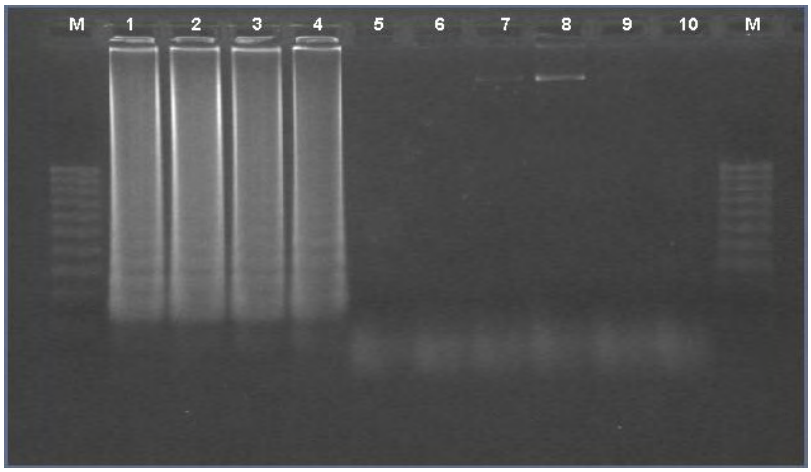
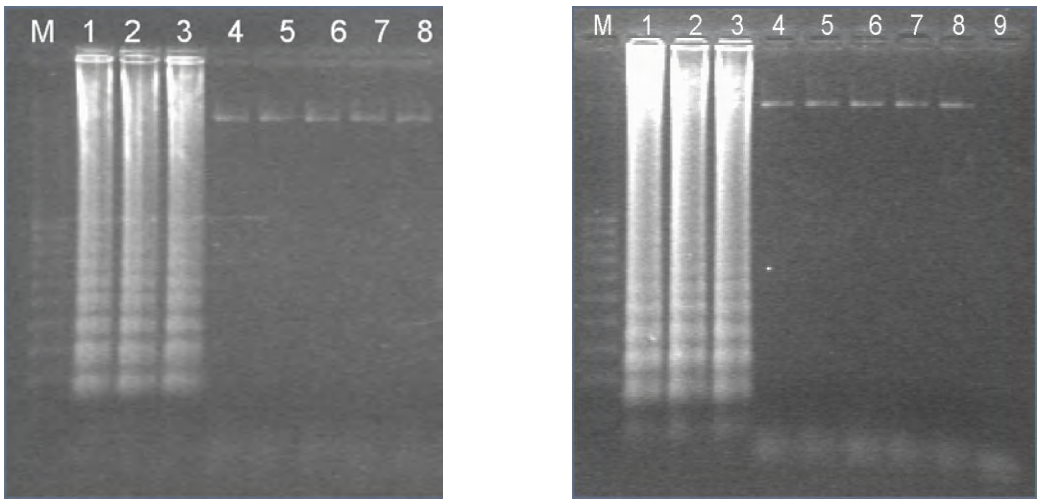


Figure 13 LAMP assay target *Lipl41* gene used to amplify DNA dilution (10^5 to 1 copy/µl reaction) of *L. interrogans* (1-6), *L. inadai* (7) and *L. biflexa* (8), *O. tsutsugamushi* DNA (9) and no template control (10). Lane M is 100 bp DNA markers.

Compare the *rrs*-LAMP assays and *lipL41*-LAMPassays

In order to compare the detection limit of both assays, spiked bacterial cell in whole blood samples were prepared as follows. The bacterial count present in an aliquot of broth culture of *L. interrogans* serovar Autumnalis strain L0551 was quantified using a Petroff-Hausser counting chamber under dark-field microscope, as previously described [40]. Ten-fold serial dilutions (10^{-1} to 10^{-6}) of the starting culture were made, and 5 µl of each dilution added to individual aliquots of 200 µl EDTA blood. These were mixed and then immediately extracted using the QIAamp blood kit (Qiagen, Germany). The detection limit of *rrs*-LAMP assays and *lipL41*-LAMPassays were equally the same that were 50 cell per reaction as shown in Figure 14 A and B respectively.



A B

Figure 14 Two LAMP assays; *rrs*-LAMP assay (A) and *lipL41*-LAMP (B) on spiked *L. interrogans* cell in 200 µl blood which equal to 5,000, 500, 50, 5, 0.5, $5x10^{-2}$, $5x10^{-3}$ and $5x10^{-4}$ cell (lane 1-8). Lane M: 100 bp DNA ladder and Lane 9: no template control (water).

The specificity and detection limit of *LipL41*-LAMP assay.

To determine the detection limit of the assay, we performed *lipL41*-LAMP assays on a serial dilution of genomic DNA extracted from pure laboratory culture of *L. interrogans* serovar Autumnalis. The DNA concentration was determined by the NanoDrop method (Thermo Fisher Scientific, USA). DNA were diluted from 100 ng downward to 0.1 fg per reaction or equivalent to $2x10^{7}$, $2x\ 10^{6}$, $2x10^{5}$, $2x10^{4}$, $2x10^{3}$, $2x10^{2}$, 20, 2 , $2x10^{-1}$, $2x10^{-2}$ and $2x10^{-3}$ copy of gene target per reaction respectively. We performed LAMP-targeting 16S rRNA (*rrs*-LAMP assays) with the same standard DNA. The detection limit of two assays were the same level which is 1 pg (or equivalent to 20 copies of gene target per reaction) as shown in Figure 15 A for *rrs*-LAMP assays and 15 B for *lipL41*-LAMP assays. Both assays could not amplify non-pathogenic leptospira (*L.biflexa*). While *rrs*-LAMP assays could amplify *Leptospira* spp. both pathogenic and intermediate group, *lipL41*-LAMP assays could not amplify intermediate group *Leptospira* spp. (*L. inadai*).

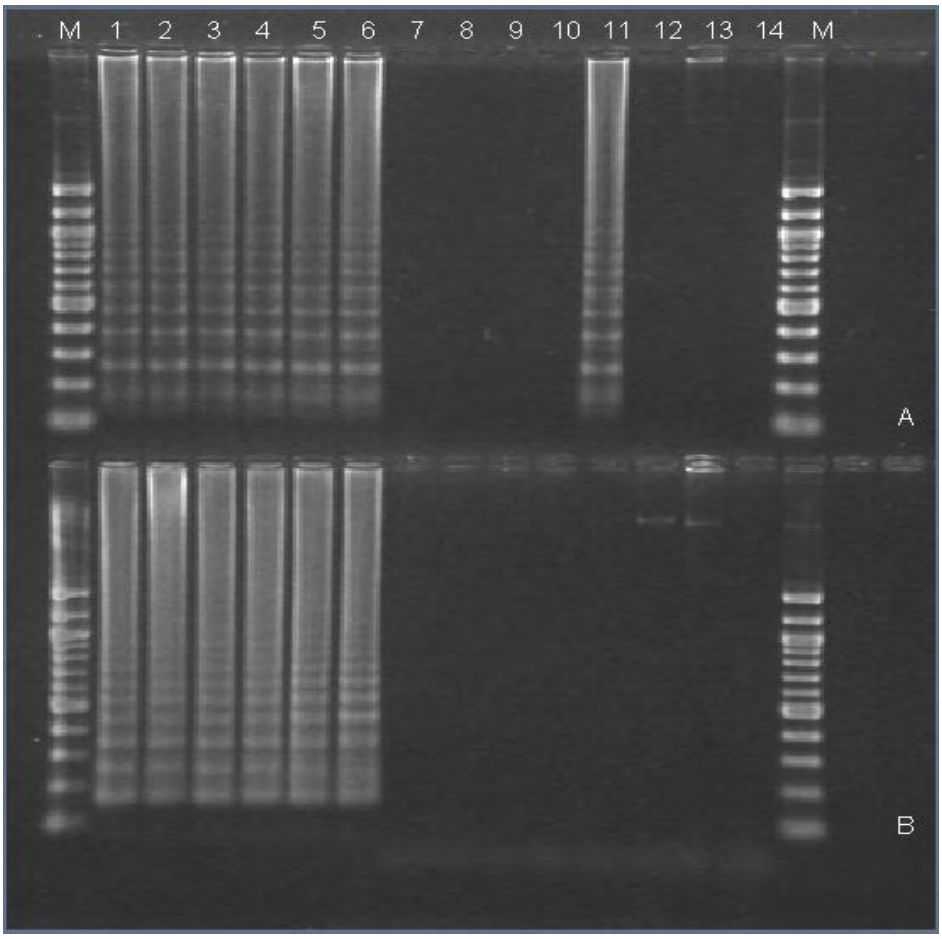


Figure 15 Two LAMP assays; *rrs*-LAMP assay (A) and *lipL41*-LAMP (B) on serial dilution of genomic DNA extracted from pure laboratory culture of *L. interrogans* of 10 serial dilution (lane 1-10) from 100 ng to 0.1 fg which equivalent to $2x10^{6}$ to $2x10^{-3}$copy/µl reaction. Lane 11: *L. inadai* DNA. Lane 12: *L. biflexa* DNA. Lane 13: human DNA. *Lane* 14: no template control (water).

Clinical validation

All 266 sample including 133 cases and 133 samples were evaluated. The result were recorded. The example of the results on 2% agarose gel electrophoresis was demonstrated in Figure 16

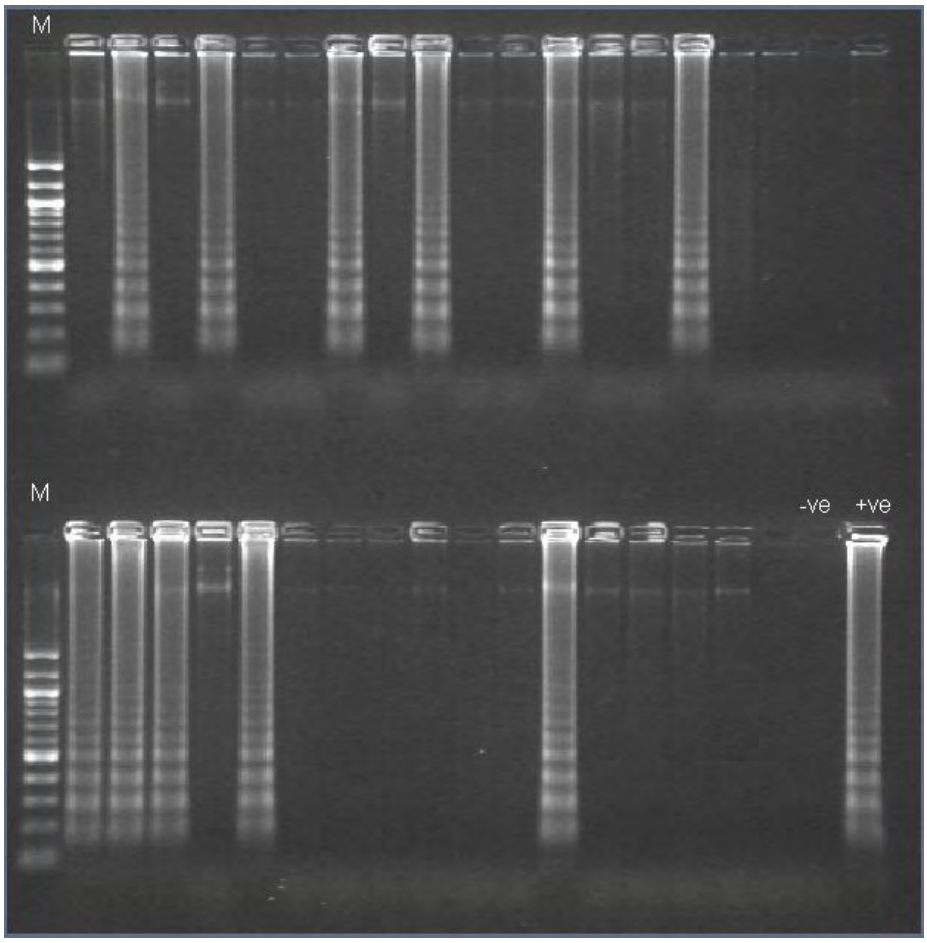


Figure 16 Example of *rrs*-LAMP assays on individual DNA sample with *L. interrogans* DNA as positive control (+ve) and No template control (-ve)

Data analysis

Diagnostic sensitivity and specificity of *rrs* LAMP.

In total, 133 patients with laboratory confirmed leptospirosis (cases) and 133 patients who did not have leptospirosis (controls) were evaluated. Patient recruitment and diagnostic testing are summarized in Figure 17. Of 133 cases, 18 patients (13%) were culture positive but MAT negative, 94 patients (71%) were MAT positive but culture negative, and 21 (16%) were both MAT and culture positive. The median (IQR, range) age was 35 years (26-46, 15-74) for cases and 42 years (29-54, 15-79) for controls (p=0.01). The median duration of illness prior to admission was 4 days (IQR 2-5 days; range 1-12 days) for cases and 6 days (IQR 3-9 days, range 0-33 days) for controls (p<0.001). The majority (120/133, 90.2%) of cases presented with $\leq$ 7 days of the onset of symptoms. Fourteen cases (11%) had received antimicrobial therapy that

would be predicted to be bacteriocidal for *Leptospira* prior to blood sample collection. The discharge diagnoses of controls were as follows: scrub typhus (n=54), other bacterial septicemia (n=8) (*Escherichia coli* (n=2), *Klebsiella pneumoniae* (n=2), *Acinetobacter baumanii* (n=1), *Corynebacterium jeikeium* (n=1), *Enterococcus* sp. (n=1), or *Streptococcus pneumoniae* (n=1)), dengue fever (n=5), murine typhus (n=4), melioidosis (n=2), HIV-related infections (n=2), other diagnoses (n=7), and unknown diagnosis (n=51). Five cases (4%) and four controls (3%) died during hospital admission.

rrs LAMP was positive in 58 of 133 cases (DSe 43.6; 95% CI: 35.0-52.5), and 22 of 133 controls (DSp 83.5; 95% CI: 76.0-89.3). The diagnoses for the 22 positive controls were scrub typhus (8), dengue (2), enterococcal septicemia (1), murine typhus (1), tuberculosis (1) and unknown (9). The duration of symptoms prior to presentation was significantly different between *rrs* LAMP positive cases and *rrs* LAMP negative cases (median 3 days, IQR 2-4 days, range 1-8 days versus median 4 days, IQR 3-6 days, range 1-12 days, respectively, $p < 0.001$).

Clinical evaluation of *lipL41* LAMP and comparison with *rrs* LAMP.

lipL41 LAMP was positive in 50 of 133 cases, (DSe 37.6; 95%CI: 29.3-46.4), and 13 of 133 controls (DSp 90.2; 95%CI: 83.9-94.7). The diagnoses for these 13 positive controls were scrub typhus (4), dengue (2), eosinophilic meningitis (cause unknown) (1), murine typhus (1) and unknown (5). The differences in DSe and DSp between the *rrs* and *lipL41* LAMP assays did not reach statistical significance ($p = 0.13$ and 0.06, respectively).

Concordance between the two assays was as follows. Cases: 43 were positive by both assays, 15 were positive for *rrs* alone and 7 cases were positive for *lipL41* alone; Controls: 8 were positive by both assays, 14 were positive for *rrs* alone and 5 were positive for *lipL41* alone.

Sensitivity of LAMP assays in patients with leptospiremia. Sensitivity was reanalyzed for 39 patients who were culture positive for *Leptospira* spp. *rrs* LAMP was positive in 33 patients (sensitivity 84.6; 95%CI: 69.5-94.1), and *lipL41* LAMP was positive in 29 patients (sensitivity 74.4; 95% CI: 57.9-87.0) ($p = 0.29$). Six culture-positive patients were negative by both assays, and all culture positive/LAMP negative patients were infected with *L. interrogans* serovar Autumnalis, the dominant cause of leptospirosis in Thailand during the study period.[41]

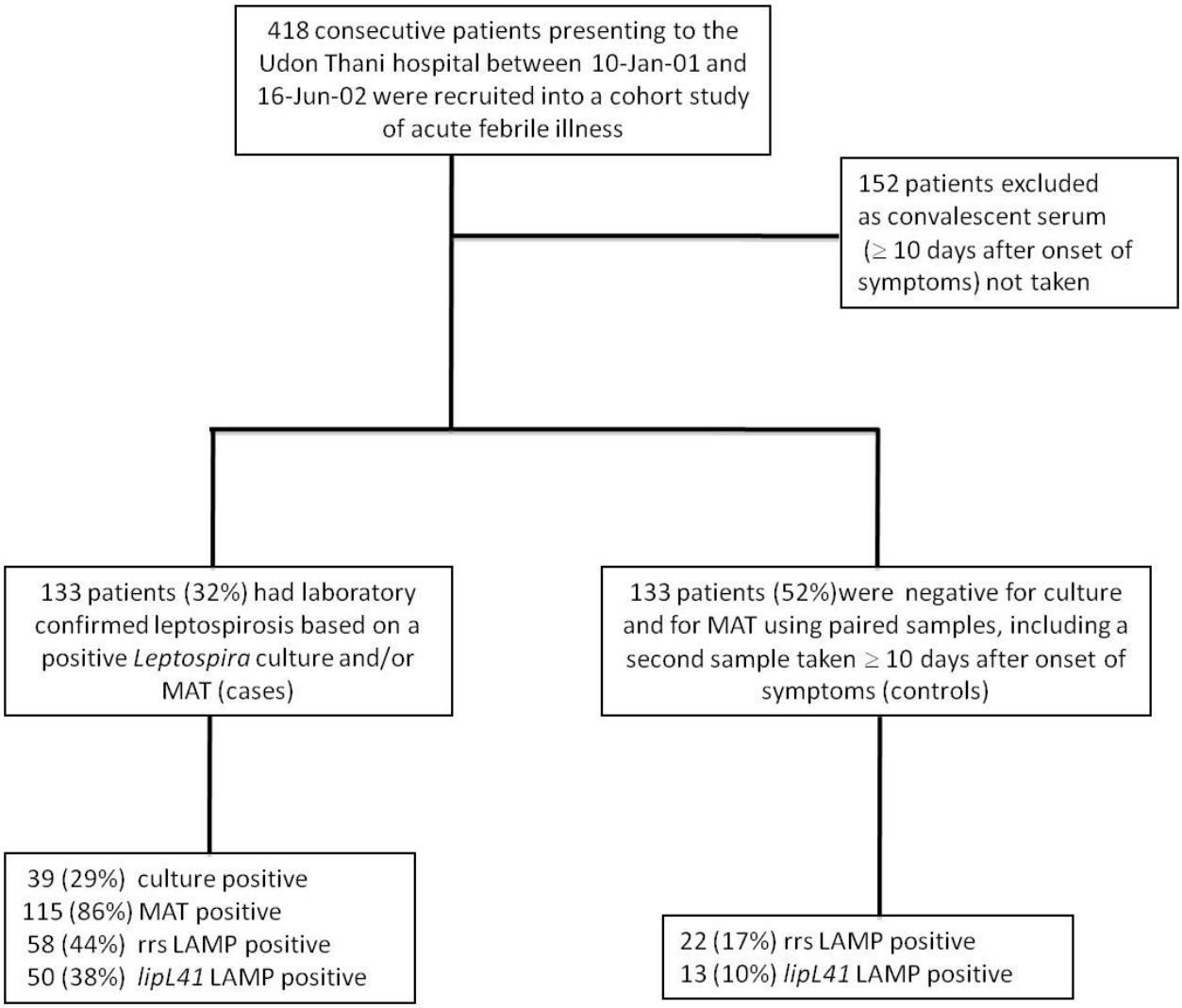


Figure 17 Flow Chart demonstrated the patient recruitment and result of laboratory tests for leptospirosis

Discussion

The ease with which LAMP can be performed to detect pathogens in clinical samples has led to its development for major infectious diseases in the developing world, including tuberculosis and malaria.[11, 42] The majority of the published literature on LAMP describes assay development together with analytical sensitivity and specificity, with relatively few studies reporting the clinical evaluation of DSe and DSp. We developed a *rrs* LAMP assay for the diagnosis of leptospirosis and demonstrated 100% analytical sensitivity and specificity, and proceeded to a clinical evaluation using samples drawn from a cohort of consecutive patients presenting with an acute febrile illness to a hospital in northeast Thailand. Having identified that the frequency of laboratory proven leptospirosis for this group was 18%, we elected to use a case-control design on the basis of cost. The DSe of *rrs* LAMP was disappointingly low (43.6%). The sensitivity of *rrs* LAMP for a subset of 39 patients who were culture-positive for *Leptospira* spp. was considerably higher (84.6%). This observation has little practical utility, however, since it is not possible to predict those patients with leptospiremia based on clinical features alone.

A LAMP assay targeting *lipL41* has been developed previously for the diagnosis of leptospirosis and was included in our clinical evaluation. Possible reasons for the lower diagnostic sensitivity of *lipL41* LAMP compared with *rrs* LAMP include the difference in lower limit of detection for the two assays (10GE/reaction and 100GE/reaction for *rrs* and *lipL41*, respectively), or infection with intermediate group *Leptospira* spp. which would not be detected by *lipL41*. The latter is not the case, however, since all 39 isolates from patients who were culture positive have been identified and none belong to the intermediate group (data not shown).

There are several possible explanations for the low DSe of both LAMP assays. A wide range of oral antimicrobial drugs are available over the counter in Thailand, and it is common for individuals to self-medicate in the period leading up to hospital admission. Many of the available antimicrobials would be predicted to be effective against *Leptospira* spp., which may lead to a false-negative LAMP result although not necessarily a negative MAT. We were aware that 14 cases had received an antimicrobial drug at the time of admission (of which 4 cases were positive by both assays), but their consumption may have been more common. The LAMP assays may have also been false negatively because the number of organisms in the sample fell below the lower limit of detection for the assay, or because of failure of primer annealing although we

consider this unlikely for *rrs* given the conserved nature of the target. Whilst further refinements of the LAMP assay may result in an improvement in the lower limit of detection and diagnostic sensitivity, we are pessimistic that molecular tests will prove highly sensitive in unselected patients in settings where antibiotic use is uncontrolled.

The DSp of both LAMP assays were lower than anticipated, and it is possible that some of the LAMP positive controls were falsely negative by culture and MAT. The sensitivity of MAT is reported to be greater than 90%,[43] and the finding in our study that 18 cases were culture positive but MAT negative highlights the imperfect sensitivity of MAT in our setting. The observation that 8 controls (with a diagnosis of scrub typhus (3), dengue (2) and unknown (3)) were positive by both *rss* and *lipL41* assays is a further hint that these patients may have been leptospiremic. In the absence of definitive evidence to support true infection rather than another explanation such as laboratory contamination, however, we must assume that these cases are false rather than true positives.

In conclusion, we have demonstrated that *rss* LAMP can identify around half of patients with leptospirosis at the time of presentation in northeast Thailand, but this is tempered by an imperfect specificity. We are unable to recommend use of LAMP in routine clinical practice until the results of additional clinical evaluations become available.

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Output of this project

1. Publication or submitted manuscript (if available)

Sonthayanon, P, Chierakul, W, Wuthiekanun,V, Thaipadungpanit, J, Kalambaheti, T Boonsilp, S, Amornchai, P, Smythe, LD, Limmathurotsakul, D, Day, NPJ and. Peacock, SJ. Accuracy of Loop-Mediated Isothermal Amplification (LAMP) for the Diagnosis of Human Leptospirosis in Thailand. *Am. J. Trop. Med. Hyg., (2011) 84(4):pp.614-620. In Press, Corrected Proof.* (เอกสารหมายแนบหมายเลข1)

2. The study has been used for teaching in Master of Science (Tropical Medicine) and Doctor of Philosophy (Tropical Medicine) of the Faculty of Tropical Medicine, Mahidol university.

2.1 Topic "How to design the LAMP primer" in Subject TMID 541 Bioinformatics

2.2 Topic "Molecular studied for Tropical Diseases" in Subject TMCD 512 Clinical Laboratory Methods for Research

3. Related activity (e.g. conference, etc.)

3.1 นำเสนอแบบบรรยาย ในการประชุมวิชาการโรคเลปโตสไปโรสิส ประจำปี 2553 "Leptospirosis in Thailand: Lessons learned and preparedness for the next outbreak" โดยชมรมเลปโตสไปโรซีสประเทศไทย ในหัวข้อ "LAMP for the diagnosis of human leptospirosis" ณ โรงแรมเดอะริช นนทบุรี วันที่ 9-10 สิงหาคม 2553

3.2 นำเสนอแบบโปสเตอร์ ในการประชุมประจำปี "นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว." ครั้งที่ 10 ในหัวข้อ "Rapid diagnosis of leptospirosis by loop-mediated isothermal amplification (LAMP)" ณ โรงแรมฮอลิเดย์ อินน์ รีสอร์ทรีเจ้นท์ บีช ชะอำ จังหวัดเพชรบุรี วันที่ 14 – วันที่ 16 ตุลาคม พ.ศ. 2553 (เอกสารหมายแนบหมายเลข 2)

3.3 นำเสนอแบบโปสเตอร์ในการประชุมนานาชาติ Oxford Tropical Medicine Network Meeting หัวข้อเรื่อง "Loop-Mediated Isothermal Amplification (LAMP) for the Diagnosis of Human Leptospirosis in Thailand" ณ เมืองเวียงจันทน์ สาธารณรัฐประชาธิปไตยประชาชนลาว วันที่ 13-16 กุมภาพันธ์ 2554 (เอกสารหมายแนบหมายเลข 3)