Rev. Inst. Med. Trop. Sao Paulo
2016;58:64
http://dx.doi.org/10.1590/S1678-9946201658064

ORIGINAL ARTICLE

COMPARISON OF 16S rRNA-PCR-RFLP, LipL32-PCR AND OmpL1-PCR METHODS IN THE DIAGNOSIS OF LEPTOSPIROSIS

Tülin GÜVEN GÖKMEN(2), Ayben SOYAL(1), Yıldız KALAYCI(1), Cansu ÖNLEN(1) & Fatih KÖKSAL(1)

SUMMARY

Leptospirosis is still one of the most important health problems in developing countries located in humid tropical and subtropical regions. Human infections are generally caused by exposure to water, soil or food contaminated with the urine of infected wild and domestic animals such as rodents and dogs. The clinical course of leptospirosis is variable and may be difficult to distinguish from many other infectious diseases. The dark-field microscopy (DFM), serology and nucleic acid amplification techniques are used to diagnose leptospirosis, however, a distinctive standard reference method is still lacking. Therefore, in this study, we aimed to determine the presence of *Leptospira* spp., to differentiate the pathogenic *L. interrogans* and the non-pathogenic *L. biflexa,* and also to determine the sensitivity and specificity values of molecular methods as an alternative to conventional ones. A total of 133 serum samples, from 47 humans and 86 cattle were evaluated by two conventional tests: the Microagglutination Test (MAT) and the DFM, as well as three molecular methods, the 16S rRNA-PCR followed by Restriction Fragment Lenght Polymorphism (RFLP) of the amplification products 16S rRNA-PCR-RFLP, LipL32-PCR and OmpL1-PCR. In this study, for *L. interrogans*, the specificity and sensitivity rates of the 16S rRNA-PCR and the LipL32-PCR were considered similar (100% versus 98.25% and 100% versus 98.68%, respectively). The OmpL1-PCR was able to classify *L. interrogans* into two intergroups, but this PCR was less sensitive (87.01%) than the other two PCR methods. The 16S rRNA-PCR-RFLP could detect *L. biflexa* DNA, but LipL32-PCR and OmpL1-PCR could not. The 16S rRNA-PCR-RFLP provided an early and accurate diagnosis and was able to distinguish pathogenic and non-pathogenic *Leptospira* species, hence it may be used as an alternative method to the conventional gold standard techniques for the rapid disgnosis of leptospirosis.

KEYWORDS: Leptospirosis; LipL32-PCR; OmpL1-PCR; 16S rRNA-PCR-RFLP.

INTRODUCTION

Leptospirosis is a zoonotic infection caused by spirochetes of the *Leptospira* genus that are usually transmitted through direct contact via injured skin or the mucosal membrane[1]. Infection may be asymptomatic, or cause symptoms that can vary from a mild flu-like illness to a severe form called Weil's disease in which patients present with jaundice and acute kidney injury[2]. Severe forms of leptospirosis can have pulmonary hemorrhage and cardiac impairment. The estimated number of leptospirosis cases is difficult and virtually overlooked due to missed or delayed diagnosis[3]. Thus, microbiological examination of clinical samples such as urine, blood and cerebrospinal fluid is important for rapid diagnosis and treatment. Dark-field microscopy (DFM), Microagglutination Test (MAT) and Polymerase Chain Reaction (PCR) are commonly used for the diagnosis of leptospirosis[4].

Although conventional methods are usually preferred in diagnosis, their advantages and disadvantages should be evaluated. Culture methods have high specificity, and culture methods coupled to MAT have been chosen as reference tests for diagnosis, but their sensitivities are low and, in the case of culture methods, it may take up to eight weeks to a final result, with weekly inspections and examinations[5,6]. Direct dark field microscopy provides rapid diagnosis of leptospirosis, but it requires experienced staff because fibrin or protein threads which may show Brownian motion may lead to misinterpretation[1]. MAT is evaluated as a reference method for diagnosis in various studies. However, MAT is based on the use of live *Leptospira,* and it is generally performed by reference laboratories[1,7]. The Enzyme-linked Immunosorbent Assay (ELISA) is cheaper and easier to use, but sensitivities range from 28 to 72% during the acute-phase of illness. On the other hand, the sensitivity of these assays may be less than 25% in patients during the first week of critical illness[8]. However, in some studies, IgM-ELISA has been used as the reference method[7].

(1) Cukurova University, Medical Faculty, Department of Microbiology. 01330 Adana, Turkey. E-mails: tulinguven01@hotmail.com; aybensoyal@hotmail.com; yldzozdmr@hotmail.com; cansuonlen@hotmail.com; fkoksal@cu.edu.tr

(2) Cukurova University, Ceyhan Veterinary Faculty, Department of Microbiology. 01330, Adana, Turkey. E-mail: tulinguven01@hotmail.com

Correspondence to: Tülin Güven Gökmen, Cukurova University, Ceyhan Veterinary Faculty, Department of Microbiology. Adana, Turkey. Tel: +90 322 338 60 60-3480; E-mail: tulinguven01@hotmail.com

Recently, ELISA, Dual Path Platform and dipstick methods that are able to detect IgM anti-leptospira have been developed[8,9,10,11]. These methods are rapid, inexpensive and portable. Early diagnosis of leptospirosis is critical for the initiation of appropriate treatment; however, the available reference serological test cannot detect antibodies until nearly a week after the onset of symptoms. Although these portable assays have high sensitivities and specificities, they should be confirmed by other diagnostic test such as MAT, culture or PCR-based assays[12,13].

PCR-based assays require well-equipped laboratories with experienced staff and as they present high costs, these methods are inappropriate for routine use in public hospitals and laboratories in developing countries. PCR methods provide rapid results in contrast to the time consuming culture and MAT[13,14]. They are also used as confirmatory tests due to their higher sensitivities and specificities in the diagnosis of leptospirosis[15]. PCR-based tests have focused on both, the universal 16S rRNA and some surface proteins such as OmpL1, LipL32, LipL36 and LipL41[14,16,17].

The 16S rRNA-PCR is a well-established PCR method targeting the 16S ribosomal RNA subunit. It is useful for the direct diagnosis of leptospirosis, is highly sensitive and specific, and able to detect approximately 10 genome equivalents (GE)/mL of whole blood, identifying *L. interrogans* and *L. biflexa* serovars. These serovars are distinguished by DNA sequencing or Restriction Fragment Length Polymorphism (RFLP) of the PCR product[14,17,18]. LipL32-PCR targeting the LipL32 surface protein gene region is only able to amplify *L. interrogans*. This method detects 100 leptospira/mL of plasma, serum or whole blood, and 0.7 genome equivalents (GE)/reaction of paraffin-embedded tissues[19,20,21]. The OmpL1-PCR targeting the OmpL1 outer membrane protein can amplify and distinguish seven groups of *L. interrogans* (Intergroup A, Intergroup B, Borgpeter, Kirschner, Santarosai, Noguchii and Weilii) by using species-specific PCR primer sets[22].

In this study, we have determined the presence of *Leptospira* spp., distinguished *L. interrogans* and *L. biflexa* and measured the sensitivity and specificity of 16S rRNA-PCR-RFLP, OmpL1-PCR and LipL32-PCR as alternatives to the conventional methods.

MATERIAL AND METHODS

Sample collection

One hundred and thirty-three serum samples were obtained from 47 humans and 86 cattle. Human serum samples were collected from patients who attended the *Cukurova* University Hospital and the *Adana* State Hospital presenting with leptospirosis symptoms characterized by fever, jaundice, hepatosplenomegaly, hematuria and kidney failure. Serum samples were collected from cattle with anorexia, fever, jaundice, and hematuria in industrial dairy farms of the *Adana* province. From all of the subjects, 5 mL of blood were collected aseptically and distributed into two sterile tubes, one dry and the other containing 500 µL of sodium oxalate at pH 8.0. The blood in the dry tube was used to perform the MAT. The tube containing sodium oxalate solution was centrifuged at 3,000 x g for 5 min and 20 µL of the supernatant were used for the dark field microscopy. The remaining serum was stored at -20 °C for the PCR tests.

Dark field microscopy (DFM)

For DFM, 20 µL of the supernatant were transferred into a new, clean slide and a cover slip was placed over it. The preparation was examined under a dark field microscope (Carl Zeiss, Germany). The remaining supernatant was then spun at 4,000 x g for 20 min and a wet sample was prepared with a drop of the sediment for the examination under the dark field microscope. The sample was reported negative if no spirochetes were observed after screening approximately 100 fields in each preparation[23]. Spirochetes determined by dark field microscopy were identified and distinguished as serovars by the MAT test.

Microagglutination Test (MAT)

Serial two-fold dilutions of the sera (1:50-1:3,200) were mixed with equal volumes of viable *L. biflexa patoc I; L. interrogans serovar hardjo; L. interrogans serovar Canicola* (Hond Utrecth IV strain)*; L. interrogans serovar Australis* (Bratislava Jez Bratislava strain)*; L. interrogans serovar hepdomadis; L. interrogans serovar ichterohemmoragica* (RGA strain)*; L. kirschneri serovar grippotyphosa* (Moskva strain)*; L. interrogans serovar pomona* (Pomona strain) and *L.interrogans serovar hardjo* (Hardjoprajitno strain) in a 96 well microtiter plate. After incubation at 30 °C for 2 h, samples were examined by MAT and DFM. MAT titers ≥ 1:400 were considered positive in single serum samples of humans and cattle[15,24].

PCR detection

For the PCR assays, genomic DNA was extracted from the 133 serum samples by using the QIAGEN DNeasy Blood & Tissue kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's instructions. The DNA concentration was estimated in a spectrophotometer (PG instrument Ltd, Lutterworth, England).

Amplification of the 16S rRNA gene region

Nested PCR assays were performed to amplify the 16S rRNA subunit as previously described[14], yielding a 525 bp PCR product after the first round of amplification. The first round primers were 5'-GGCGGCGCGTCTTAAACATG-3' and 5'-GTCCGCCTACGCACCCTTTACG-3. The second round of amplification was carried out using 1 µL of the first PCR product as the template for the second round, and the primers 5'-CAAGTCAAGCGGAGTAGCAA-3' and 5'-TAACCTGCTGCCTCCCG TA-3'. The second amplification product was 289 bp. Amplification products were subjected to horizontal electrophoresis on 1.2% agarose gels. Amplification products were digested with the *Apo*I enzyme resulting in a DNA pattern that could distinguish *L. biflexa* and *L. interrrogans.*

Amplification of the OmpL1 gene region

The OmpL1-PCR was performed with two pair of primers to determine different serotypes of L. *interrogans*. The intergroup A primers (5'-CTACTGGCGGCTTGATC AAC-3' and 5'-CTGGATCTGTTCCGTCTGCGATC-3') were used to obtain 396 bp PCR products. The intergroup B primers (5'-CTTGATAGAACCACTGGTGGTGCC-3' and

5'-TGGATCGGTTCCAT CTGCTCAG-3') were used to obtain 406 bp products, as previously described[22]. Amplification products were subjected to horizontal electrophoresis on 1.2% agarose gels.

Amplification of the LipL32 gene region

Nested LipL32-PCR is able to amplify only the *L. interrogans* group according to a previously described protocol[16,25]. The primers used for first amplification were 5'-CTAAGTTCATACCGTGATTT-3' and 5'-TCTGACGCGACTA AGTAAT-3' yielding a 859 bp product. The second round of amplification was carried out using 1 µL of the first PCR product as the template, and the primers 5'-GACGGTTTAGTCGATGGAAA C-3' and 5'-GGGAAAACAGACCAACAGA-3'. Amplification products were subjected to horizontal electrophoresis on 1.2% agarose gels.

Statistical analysis

The results were evaluated and the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and 95% Confidence Interval (CI) were calculated.

RESULTS

Different tests were employed for the diagnosis of leptospirosis in serum samples of humans and cattle. All of the 133 serum samples suspected of leptospirosis and reference strains were tested by MAT, DFM, 16S rRNA-PCR, LipL32-PCR and OmpL1-PCR.

Among the 133 serum samples, 90 (67.6%) were found positive by MAT: 21 (44.6%) from humans and 69 (80%) from cattle. MAT was performed in all serum samples at dilutions between 1:50 and 1:3,200. More than one serovar was detected in 64 samples (48.1%). However, only one serovar with a titer ≥ 1:400 was considered as an infecting serovar.

In DFM, spirochetes were observed in 56 (42.1%) samples: 22 (39.2%) from humans, 34 (60.8%) from cattle. Of the 56 positive samples, 35 were concordant with MAT results and *L. interrogans* was determined in 21 samples (Table 1).

Ninety two samples (69.17%) of the 133 serum samples were positive by the 16S rRNA PCR: 35 from humans and 57 from cattle. The positive 16S rRNA-PCR samples were also positive by at least one of the other methods. The 92 positive samples yielded the 289 bp fragment and these amplification products were digested with the *Apo*I restriction enzyme to distinguish *L. interrogans* (289 bp) and *L. biflexa* (89 bp -200 bp). *L. interrogans* was detected in 77 samples (27 from humans and 50 from cattle). Ninety of the 92 samples (97.82%) were concordant with MAT. The remaining two samples were negative by MAT but positive by DFM.

Seventy seven samples were found to contain *L. interrogans* as they were positive by LipL32-PCR, and all of them yielded the 497 bp product.

The OmpL1-PCR has found positive results in 67 serum samples. Intergroup A (396 bp) and Intergroup B (406 bp) specific fragments were found in 14 serum samples from humans and 53 serum samples from cattle, respectively (Fig. 1, Table 2).

Table 1
Determination of *Leptospira* spp. and positive values over total samples (n = 133), according to the microagglutination test (MAT)

	Microaggltination Test (MAT)	
	Positive samples/ Total	(%)
L. biflexa patoc I	26/133	19.55
L. interrogans serovar hardjo	30/133	22.55
L. interrogans serovar canicola	2/133	1.50
L. interrogans serovar bratislava	6/133	4.51
L. interrogans serovar hepdomadis	1/133	0.75
L. interrogans serovar ichterohemmoragica	5/133	3.76
L. interrogans serovar grippotyphosa	14/133	10.53
L. interrogans serovar pomona	6/133	4.51
TOTAL	90/133	67.66

Molecular and conventional methods were evaluated regarding the sensitivity, specificity, positive and negative predictive values, using a 95% CI. The parameters were calculated based on 16S rRNA-PCR results for *L. interrogans* (Table 3).

DISCUSSION

In the present study, MAT, DFM and three PCR methods (16S rRNA-PCR-RFLP; LipL32-PCR and OmpL1-PCR) were performed for the diagnosis of leptospirosis. PCR assays have higher specificities and sensitivities, and they can be acomplished more rapidly, are simpler and more reliable in diagnosing leptospirosis, offering several advantages in comparison with the conventional diagnostic methods[26,27,28].

According to the Leptospirosis Burden Epidemiology Reference Group Criteria, a positive MAT is defined as a MAT titer ≥ 1:400 in human and cattle serum samples[15,24]. MAT sensitivity and specificity were found to be 82.43% and 94.92%, respectively (Table 3). Other studies have demonstrated that the sensitivity is in the range of 86.9% to 96.2%[29,30,31,32]. Thus, our findings remain slightly below other reported data. Furthermore, in Turkey, there is only one reference laboratory (Etlik Spirochetes Disease Diagnostic Laboratory) that performs MAT and culture tests on both, animals and human samples.

The DFM method was used to evaluate human and cattle serum samples. Detection by DFM had a sensitivity of 27.27% in this study (Table 3), which is consistent with previous reports[33]. However, there are studies in which the sensitivity of DFM varied from 61% to 93.3%[23,34]. Therefore, the discrepancy between our findings and those of previous studies may be explained by the fact that, in this study, serum samples were obtained during different stages of the disease.

A comparison between MAT and DFM has shown that the sensitivity of MAT was higher than the one found for DFM[35]. The DFM assay has a low sensitivity because approximately 10^4 leptospires/mL of blood are necessary in each of the cells per field to be visible[36].

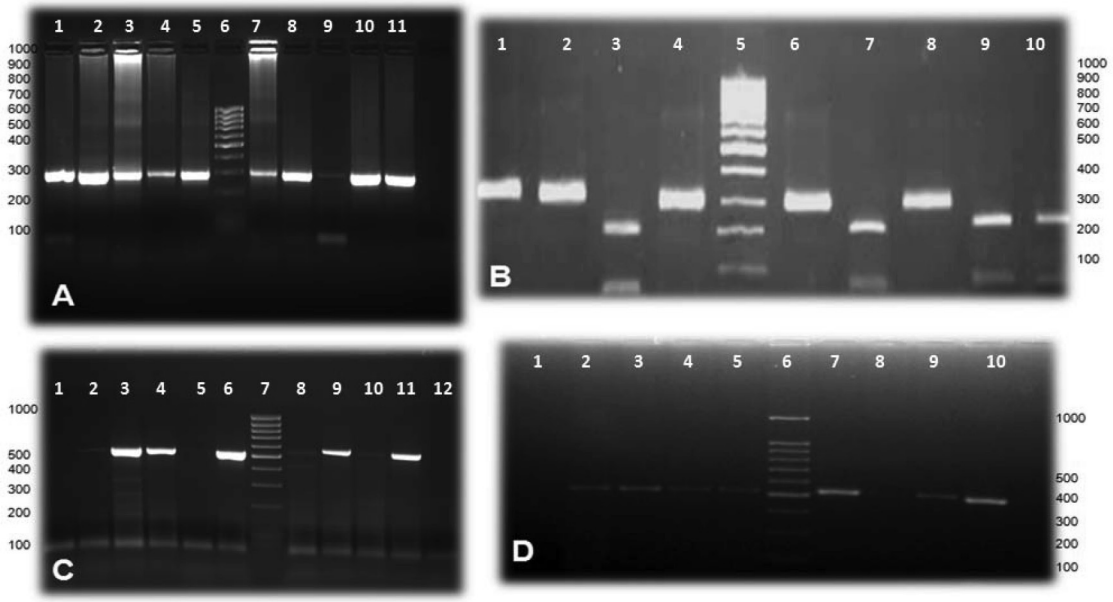

Fig.1 - **(A)** 16S rRNA-PCR products. Lane 6: 100 bp DNA ladder; Lane 1-10: 289 bp product. **(B)** 16S rRNA-PCR-RFLP products. Lane 5: 100 bp DNA ladder; Lane 1-2-4-6-8: RFLP with *Apo*I for *L. interrogans* (289 bp); Lane 3-7-9-10: RFLP with *Apo*I for reference *L. biflexa* (200 bp and 89 bp products). **(C)** LipL32-PCR products. Lane 7: 100 bp DNA ladder; Lane 1-12: 497 bp product. **(D)** OmpL1-PCR products. Lane 6: 100 bp DNA ladder; Lane 1-10: 406 bp product.

Table 2
Positive values over total samples (133) according to the tested methods

	DFM *	MAT	16S rRNA	LipL32	OmpL1
L. interrogans	21/133	64/133	77/133	77/133	67/133
L. biflexa	14/133	26/133	15/133	-	-
TOTAL	35/133	90/133	92/133	77/133	67/133

*Spirochetes determined by dark field microscopy (DFM) were identified and distinguished as serovars by the microagglutination test (MAT). 16S rRNA, LipL32 and OmpL32 are PCR techniques.

Three PCR-based methods have been used for the diagnosis of *L. interrogans.* The 16S rRNA-PCR-RFLP, is known to have high sensitivity and specificity, and is considered as an alternative approach to the conventional gold standard diagnostic methods. In the present study, the 16S rRNA-PCR-RFLP has identified all of the positive samples (100% of sensitivity, Table 3), which were also positive by at least one of the other conventional and molecular diagnostic methods.

The sensitivity of the 16S rRNA-PCR assay was 100%, and the specificity was 100% (Table 3). The sensitivity and specificity of the LipL32-PCR were 98.68% and 98.25%, respectively (Table 3). It has been reported that 16S rRNA and LipL32 genes were specific for *L. interrogans.* Thus, PCR assays targeting the 16S rRNA subunit and LipL32 genes have been used to detect *L. interrogans*[14,16,17,37,38]. The OmpL1-PCR can detect and differentiate among seven Leptospira species representing the majority of the currently known pathogenic strains[23]. The OmpL1-PCR was less sensitive in this study (sensitivity of 87.01%, Table 3) than the other PCR methods.

In this study, 16S rRNA-PCR-RFLP and MAT were used to detect non-pathogenic strains. Albeit *L. biflexa* has been isolated from mammalian hosts on occasion, no pathological effects have been detected so far. Nevertheless, it remains a major cause of misdiagnosis. In DFM,

Table 3
Sensitivity and specificity values of all the methods used in this study

	Positive samples	Sensitivity	95% CI	Specificity	95% CI*	PPV**	NPV***
DFM	21	27.27%	17.75-38.62	100%	93.56-100	100%	50%
MAT	64	82.43%	71.83-90.29	94.92%	85.83-98.88	95.31%	81.16%
16S rRNA-PCR	77	100%	95.28-100	100%	93.56-100	100%	100%
LipL32-PCR	77	98.68%	92.86-99.78	98.25%	90.57-99.71	98.68%	98.25%
OmpL1-PCR	67	87.01%	77.41-93.58	100%	93.56-100	100%	84.85%

*: Confidence interval, **: Positive Predictive Value, ***: Negative Predictive Value

L. biflexa can lead to false positive results particularly in symptomatic patients. Regarding MAT, during the acute phase of *Leptospira* spp. infections, paradoxical reactions may occur with non-pathogenic serovars leading to potential misdiagnosis with the pathogenic *Leptospira* spp. strains[39]. Therefore, the elimination of misdiagnosis can be provided by using the 16S rRNA-PCR-RFLP.

We evaluated both human and cattle samples with respect to sensitivity and specificity of 16S rRNA-PCR-RFLP (100% and 100%, respectively), LipL32-PCR (98.68% and 98.25%, respectively) and OmpL1-PCR (87.01% and 100%, respectively). This strategy was chosen because a method to be used as an alternative to the conventional gold standard ones in future studies should be able to evaluate samples from different species.

The sensitivity and specificity values for both human and cattle samples have been compared using 16S rRNA-PCR-RFLP, LipL32-PCR and OmpL1-PCR. The sensitivity and specificity of the 16S rRNA-PCR-RFLP were (100% and 100%) and (100% and 100%) for cattle and human samples, respectively. Furthermore, the sensitivity and specificity of the LipL32-PCR were (100% and 96.3%), (100% and 51.85%); and for the OmpL1-PCR were (97.2% and 100%), and (91.67% and 100%) for cattle and human samples, respectively. While the sensitivity of 16S rRNA-PCR and LipL32-PCR were quite high and similar (100% in both cases), the specificity of LipL32-PCR was very low in serum samples (51.85%). Regarding the OmpL1-PCR, the sensitivity of 91.67% for human samples was lower than the 100% of the 16S rRNA-PCR, though the specificity has been 100% for both, cattle and human samples.

In conclusion, the 16S rRNA-PCR-RFLP has provided early and rapid diagnosis of pathogenic and non-pathogenic *Leptospira* species in blood samples. Thus, we recommend that the 16S rRNA-PCR-RFLP should be used rather than the two conventional tests (MAT and DFM) and the other two molecular methods (LipL32-PCR and OmpL1-PCR). The 16S rRNA-PCR-RFLP may be used as an alternative assay to the conventional gold standard diagnostic methods for the rapid and reliable identification of *Leptospira* spp.

FUNDING

This work was supported by the *Cukurova* University Scientific Research Projects Unit [TF2009D1].

REFERENCES

1. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001;14:296-326.

2. Ganoza CA, Matthias MA, Saito M, Cespedes M, Gotuzzo E, Vinetz JM. Asymptomatic renal colonization of humans in the peruvian Amazon by *Leptospira*. PLoS Negl Trop Dis. 2010;4:e612.

3. Plank R, Dean D. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. Microbes Infect. 2010;2:1265-76.

4. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control. [cited 2015 Jun 20]. Available from: http:// www.who.int/csr/don/en/ WHO_CDS_CSR_EPH_2002.23.pdf.

5. Smythe LD, Smith IL, Smith GA, Dohnt ML, Symonds ML, Barnett LJ, et al. A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. BMC Infect Dis. 2002;2:13.

6. Limmathurotsakul D, Turner EL, Wuthiekanun V, Thaipadungpanit J, Suputtamongkol Y, Chierakul W, et al. Fool's gold: why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. Clin Infect Dis. 2012;55:322-31.

7. Niloofa R, Fernando N, de Silva LN, Karunanayake L, Wickramasinghe H, Dikmadugoda N, et al. Diagnosis of leptospirosis: comparison between microscopic agglutination test, IgM-ELISA and IgM rapid immunochromatography test. PLoS One. 2015;10:e0129236.

8. Croda J, Ramos JGR, Matsunaga J, Queiroz A, Homma A, Riley LW, et al. *Leptospira* immunoglobulin-like proteins as a serodiagnostic marker for acute leptospirosis. J Clin Microbiol. 2007;45:1528-34.

9. Félix SR, Hartwig DD, Argondizzo AP, Silva EF, Seixas FK, Neto AC, et al. Subunit approach to evaluation of the immune protective potential of leptospiral antigens. Clin Vaccine Immunol. 2011;18:2026-30.

10. Larentis AL, Nicolau JF, Esteves GS, Vareschini DT, de Almeida FV, dos Reis MG, et al. Evaluation of pre-induction temperature, cell growth at induction and IPTG concentration on the expression of a leptospiral protein in *E. coli* using shaking flasks and microbioreactor. BMC Res Notes. 2014;7:671.

11. Nabity SA, Ribeiro GS, Aquino CL, Takahashi D, Damião AO, Gonçalves AH, et al. Accuracy of a dual path platform (DPP) assay for the rapid point-of-care diagnosis of human leptospirosis. PLoS Negl Trop Dis. 2012;6:e1878.

12. Galloway RL, Hoffmaster AR. Optimization of LipL32 PCR assay for increased sensitivity in diagnosing leptospirosis. Diagn Microbiol Infect Dis. 2015;82:199-200.

13. World Health Organization. Leptospirosis. [cited 2015 June 10]. Available from: http:// www.who.int/zoonoses/diseases/LeptospirosissurveillancE.pdf

14. Mérien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol. 1992;30:2219-24.

15. World Health Organization. Report of the second meeting of the leptospirosis burden epidemiology reference group. Geneva: WHO; 2011 [cited 2015 Aug 1]. Available from: http://apps.who.int/iris/bitstream/10665/44588/1/9789241501521_eng.pdf

16. Haake DA, Suchardhttp://jb.asm.org/content/186/9/2818.abstract - aff-3 MA, Kelley MM, Dundoo M , Alt DP, Zuerner RL. Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J Bacteriol. 2004;186:2818-28.

17. Haake DA, Martinich C, Summers TA, Shang ES, Pruetz JD, MacCoy AM, et al. Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect Immun. 1998;66:1579-87.

18. Natarajaseenivasan K, Raja V, Narayanan R. Rapid diagnosis of leptospirosis in patients with different clinical manifestations by 16S rRNA gene based nested PCR. Saudi J Biol Sci. 2012;19:151-5.

19. Bedir O, Kilic A, Atabek E, Kuskucu AM, Turhan V, Basustaoglu AC. Simultaneous detection and differentiation of pathogenic and nonpathogenic Leptospira spp. by multiplex real-time PCR (TaqMan) assay. Pol J Microbiol. 2010;59:167-73.

20. González S, Geymonat JP, Hernández E, Marqués JM, Schelotto F, Varela G. Usefulness of real-time PCR assay targeting lipL32 gene for diagnosis of human leptospirosis in Uruguay. J Infect Dev Ctries. 2013;7:941-5.

21. Noda AA, Rodríguez I, Rodríguez Y, Govín A, Fernández C, Obregón AM. High sensitive PCR method for detection of pathogenic *Leptospira* spp. in paraffin-embedded tissues. Rev Inst Med Trop Sao Paulo. 2014;56:411-5.

22. Reitstetter RE. Development of species-specific PCR primer sets for the detection of Leptospira. FEMS Microbiol Lett. 2006;264:31-9.

23. Sharma KK, Kalawat U. Early diagnosis of leptospirosis by conventional methods: one-year prospective study. Indian J Pathol Microbiol. 2008;51:209-11.

24. Faine S, Adler B, Bolin C, Perolat P. Leptospira and leptospirosis. 2nd ed. Melbourne: MediSci; 1999.

25. Bomfim MR, Barbosa-Stancioli EF, Koury MC. Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. Vet J. 2008;178:251-6.

26. Waggoner JJ, Balassiano I, Abeynayake J, Sahoo MK, Nohamed-Hadley AM, Liu Y, et al. Sensitive real-time PCR detection of pathogenic *Leptospira* spp. and a comparison of nucleic acid amplification methods for the diagnosis of leptospirosis. PLoS One. 2014;9:e112356.

27. Bourhy YP, Bremonthttp://jcm.asm.org/content/49/6/2154.full - aff-1 S, Zinini F, Giry C, Picardeau M. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J Clin Microbiol. 2011;49:2154-60.

28. Cerqueira GM, Picardeau M. A century of *Leptospira* strain typing. Infect Genet Evol. 2009;9:760-8.

29. Thaipadungpanit J, Chierakul W, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Boonslip S, et al. Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipl32 genes for human leptospirosis in Thailand: a case-control study. PLoS One. 2011;6:e16236.

30. Wuthiekanun V, Chierakul W, Limmathurotsakul D, Smythe LD, Symonds ML, Dohnt MF, et al. Optimization of culture of Leptospira from humans with leptospirosis. J Clin Microbiol. 2007;45:1363-5.

31. Phimda K, Hoontrakul S, Suttinont C, Chareonwat S, Losuwanaluk K, Chueasuwanchai S, et al. Doxycycline versus azithromycin for treatment of leptospirosis and scrub typhus. Antimicrob Agents Chemother. 2007;51:3259-63.

32. Wuthiekanun V, Sirisukkarn N, Daengsupa P, Sakaraserane P, Sangkakam A, Chierakul W, et al. Clinical diagnosis and geographic distribution of leptospirosis, Thailand. Emerg Infect Dis. 2007;13:124-6.

33. Jaiswal NK, Chandrasekaran S, Padmavathy BK. Dark field microscopy an important conventional technique for the early diagnosis of leptospirosis. Int J Curr Microbiol App Sci. 2015;4:718-22.

34. Chandrasekaran S, Gomathi S. A standard screening test for the early and rapid diagnosis of leptospirosis. Indian J Med Microbiol. 2004;22:23-7.

35. Krishna SV, Joseph S, Ambily R, Mini M. Evaluation of dark field microscopy, isolation and microscopic agglutination test for the diagnosis of canine leptospirosis. Int J Pharm Biol Sci. 2012;2:85-9.

36. Bourhy P, Bremont S, Zinini F, Giry C, Picardeau M. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J Clin Microbiol. 2011;6:2154-6.

37. Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvão Reis M, et al. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun. 2001;69:4958-68.

38. Tansuphasiri U, Chanthadee R, Phulsuksombati D, Sangjun N. Development of a duplex-polymerase chain reaction for rapid detection of pathogenic *Leptospira*. Southeast Asian J Trop Med Public Health. 2006;37:297-308.

39. World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control. Geneva: WHO; 2011. [cited 2015 Aug 3]. Available from: http://www.who.int/csr/don/en/WHO_CDS_CSR_EPH_2002.23.pdf

Received: 26 June 2015
Accepted: 28 March 2016