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Development of an immunochromatographic test with anti-LipL32-coupled gold nanoparticles for *Leptospira* detection

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SUMMARY

Detection of antibody specific to *Leptospira* by various immunological techniques has been used for leptospirosis diagnosis. However, the sensitivity of antibody detection during the first few days after infection is low. Molecular techniques are suggested to provide earlier diagnosis than antibody detection, but a rapid and easy to perform assay for *Leptospira* antigen detection would provide an additional useful tool for disease diagnosis. In this study, we coupled gold nanoparticles with antibody to LipL32, a protein commonly found in pathogenic *Leptospira*. This coupled gold reagent was used in the immunochromatographic strip for *Leptospira* detection. We demonstrated that the sensitivity of *Leptospira* detection by this strip was 10^3 ml^{-1}. There was no positive result detected when strips were tested with non-pathogenic *Leptospira*, *Staphylococcus aureus*, *Streptococcus* group B, *Acinetobacter baumannii, Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterococcus faecalis* or *Enterococcus faecium*. These data suggest that gold nanoparticles coupled with antibody to LipL32 could be used for *Leptospira* detection by a rapid test based on an immunochromatographic technique.

KEY WORDS: Leptospirosis, LipL32, Diagnosis, Immunochromatography.

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INTRODUCTION

Leptospirosis is a zoonosis caused by pathogenic *Leptospira*. Clinical manifestations presented by leptospirosis patients vary from mild to severe symptoms. In addition, patients usually present with symptoms similar to other tropical infectious diseases such as malaria and dengue virus infection. Laboratory testing is essential for proper differential diagnosis. Since the culture positive rate is very low and it takes at least one week before organisms can be observed, *Leptospira* antibody detection is commonly used for laboratory diagnosis (Bharti*et al.*2003). Various immunological techniques such as microscopic agglutination, indirect immunofluorescence and ELISA have been used for antibody detection (Appassakij *et al.* 1995; Chirathaworn *et al.,* 2007; Shekatka *et al.,* 2010; Silpasakorn *et al.,* 2011). However, the amount of antibody is usually low during the first week of infection. Paired sera are often required for demonstration of seroconversion in order to confirm the diagnosis. Molecular techniques such as PCR and real-time PCR were developed to detect *Leptospira* DNA (Ahmed *et al.,* 2009; Bourhy *et al.,* 2011). However, these techniques are relatively expensive and require special equipment.

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Leptospirosis is mostly endemic in rural areas. An easy to perform diagnostic assay will be useful for prompt diagnosis. The immunochromatographic technique has been widely used for development of the rapid diagnostic assay. We previously reported that gold nanoparticles coupled with antibody to *Leptospira interrogans* serovar Bratislava could be used for *Leptospira* detection (Chirathaworn *et al.,* 2011). We further investigated whether antibody to the specific *Leptospira* component commonly found in pathogenic *Leptospira* can be coupled to gold nanoparticles so the reagent can be used for detection of other pathogenic *Leptospira* serovars. LipL32 is a 32-kDa outer protein found only in pathogenic *Leptospira*. Moreover, this protein is the most abundant and highly immunogenic, strongly reacting with sera from leptospirosis patients (Haake *et al.,* 2000; Cullen *et al.,* 2002; Cullen *et al.,* 2005).

In this study, we coupled gold nanoparticles with antibody to LipL32, and anti-LipL32-coupled gold particles were used to develop an immunochromatographic assay for *Leptospira* detection.

MATERIALS AND METHODS

The study protocol was approved by the Ethical Committee, Faculty of Medicine, Chulalongkorn University.

Bacteria and animals

Leptospira used in this study were kindly provided by the National Institute of Animal Health, Department of Livestock Development, Thailand. All leptospires were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 30°C**.** *Staphylococcus aureus*, *Streptococcus* group B, *Acinetobacter baumannii, Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Enterococcus faecium*isolated from clinical samples were obtained from the Department of Microbiology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand.

A New Zealand white female rabbit purchased from National Laboratory Animal Center, Mahidol University was used for LipL32 immunization.

LipL32 purification

Plasmid containing *lipl32* gene (*plipl32*) was constructed as previous reported (Haake*, et al.*, 2000, Boonyod*, et al.*, 2005). LipL32 protein expression was induced in BL21 *E. coli* with 1 mM IPTG (isopropyl b-D-thiogalactopynoside). LipL32 protein was then purified using Protino® Ni-TED Resin (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) according to the manufacturer's protocol. Purified protein was observed by SDS-PAGE and Coomassie Brilliant Blue staining.

Rabbit anti-LipL32 antibody production and purification

Purified LipL32 was used to immunize a rabbit using a standard protocol. Briefly, fifty micrograms of LipL32 combined with complete Freund's adjuvant (Sigma-Aldrich, Steinheim, Germany) were injected subcutaneously after pre-immunized blood was collected. Immunizations were repeated on days 14, 28 and 56 with 50 µg LipL32 combined with incomplete Freund's adjuvant (Sigma-Aldrich). Blood was collected on day 84. Rabbit IgG was purified from immunized serum using HiTrap™ Protein G HP (GE Healthcare, Sweden) according to manufacturer's instructions. Western blot was performed to demonstrate binding of rabbit anti-LipL32 antibody to LipL32. Briefly, 500 ng of purified LipL32 was subjected to 10% SDS-PAGE and protein was then transferred to nitrocellulose. The blot was probed with rabbit anti-LipL32 antibody followed by goat anti-rabbit IgG conjugated with horse radish peroxidase (Dako, Glostrup, Denmark). The protein band was visualized by incubating the blot with ECL reagent (Amersham, Buckinghamshire, UK).

Immunochromatographic test strip preparation

Rabbit anti-LipL32 was used for *Leptospira* detection by immunochromatographic test strip. Nitrocellulose membrane (Millipore, MA, USA), sample pad, conjugate pad and absorbent pad (i+MED Laboratories, Bangkok, Thailand) were assembled into a chromatographic strip as shown in Figure 1. Three microliters of 5 mg ml^{-1} goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) were ap-

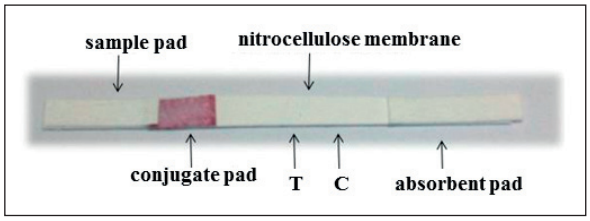

FIGURE 1 - *Composition of the immunochromatographic test strip. Gold nanoparticles coupled with antibody to LipL32 were applied on a conjugate pad. Anti-LipL32 and anti-rabbit immunoglobulin were used to treat the nitrocellulose membrane on T and C positions, respectively. A strip composed of a sample pad, a conjugate pad, a piece of nitrocellulose membrane and an absorbent pad was then assembled as described in Materials and Methods.*

plied in the control line position (C). Test line (T) was applied with 3 µl of 0.6 mg ml^{-1} of rabbit anti-LipL32. After applying antibodies, the nitrocellulose membrane was blocked with 2% non-fat milk for 30 minutes and dried at 37°C for 1 hour.

For conjugate reagent, gold nanoparticles prepared as previously reported (Chirathaworn *et al.*, 2011) were coated with rabbit anti-LipL32 and then blocked with 10% bovine serum albumin (Sigma-Aldrich) for one hour. Antibody-coated gold particles were washed and resuspended in 2 mM borate buffer (pH 9.0), 1% BSA, 1% sucrose and 0.05% sodium azide. Five microliters of antibody-coated gold suspension were then applied on a conjugate pad (i+MED Laboratories, Thailand). The conjugate pad was dried at 37°C for 30 minutes. Treated nitrocellulose membrane, sample pad, conjugate pad and absorbent pad were then attached to the polyethylene sheet as shown in Figure 1.

Detection of leptospires using anti-LipL32 immunochromatographic strips was done as follows. *Leptospira* suspension was heated at 56°C for 30 minutes followed by centrifugation at 10,000 g for 10 minutes. Fifty microliters of supernatant were then applied onto the sample pad area of the strip.

The appearance of pink lines at T and C zones indicates a positive result. The appearance of a pink line at C zone only indicates a negative result.

RESULTS

Reactivity of rabbit anti-LipL32 against LipL32

LipL32 was purified as described in Materials and Methods. Purified LipL32 protein was subjected to 10% SDS-PAGE and gel was stained with Coomassie brilliant blue (Fig. 2A). LipL32 was used to immunize a rabbit. Reactivity of rabbit anti-LipL32 against purified LipL32 was demonstrated by Western blot as shown in Figure 2B. Purified LipL32 was probed with pre-immunized rabbit serum (Fig. 2B, Lane 1), serum from a rabbit immunized with LipL32 (Lane 2) and rabbit IgG purified from a LipL32-immunized rabbit (Lane 3). Serum or purified

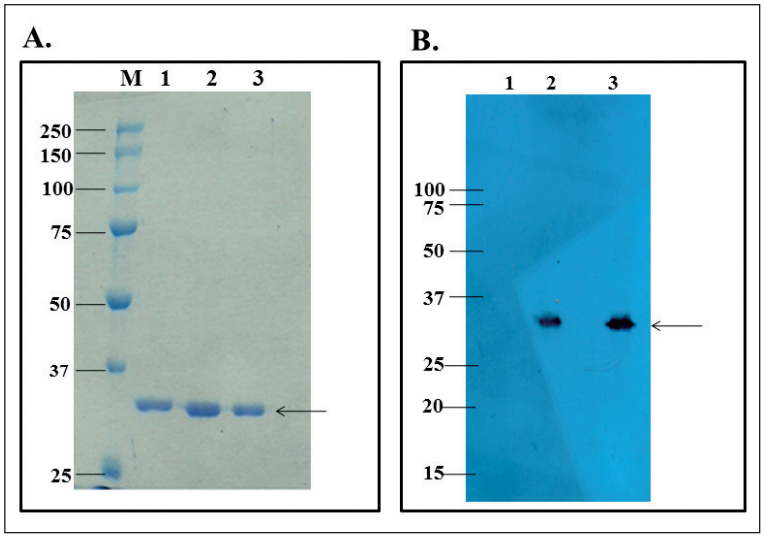

FIGURE 2 - *Reactivity of rabbit anti-LipL32 against purified LipL32 based on Western blots. A) LipL32 was purified from pLipL32 plasmid as described in Materials and Methods. Purified protein was subjected to SDS-PAGE and gel was stained with Coomassie Brilliant Blue. M lane is molecular weight markers. Lanes 1, 2, and 3 are eluates of LipL32. The arrow indicates LipL32 band. B) Western blot was performed as described in Materials and Methods. LipL32 was probed with pre-immunized rabbit serum (Lane 1), serum from a LipL-32 immunized rabbit (Lane 2) and rabbit IgG purified from LipL32-immunized rabbit (Lane 3). The arrow indicates the band of LipL32 that reacted with antisera.*

IgG from the immunized rabbit reacted with LipL32 protein. No band was detected when the blot was probed with serum from a pre-immunized rabbit.

Immunochromatographic strip for *Leptospira* detection

Purified rabbit IgG against LipL32 was used to develop immunochromatographic strips for *Leptospira* detection as follows. Various amounts (10, 30 and 50 µg ml^{-1}) of rabbit anti-LipL32 were coated on gold nanoparticles. Anti-LipL32-coated gold particles were applied on the conjugate pad and test strips were assembled as described in Materials and Methods. Fifty microliters of supernatant from heated 10^8 ml^{-1} *Leptospira interrogans* serovar Bratislava, one of the common serovars found in Thailand, were applied on the sample pad. As shown in Figure 3A, the intensity of the band was not changed when the amount of antibody was increased. In the following experiments, anti-LipL32 at 30 µg ml^{-1} was used for gold nanoparticle coupling.

***Leptospira* detection by strip test**

The strips were tested with 23 representative pathogenic *Leptospira* serovars found in Thailand and a non-pathogenic serovar. As shown in Figure 3B, positive results were observed when strips were tested with pathogenic *Leptopira* serovars Bratislava, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum, Sarmin, Sejroe, Shermani and Tarassovi (Figure 3B Strips # 1-23, respectively). There was no band detected in the test zone when non-pathogenic *Leptospira biflexa* serovar Patoc was tested (Fig. 3B, Strip #24).

Various concentrations of *Leptospira interrogans* serovar Bratislava were tested to determine limit of detection.

As shown in Figure 4, positive results were observed when the suspension of 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 leptospires ml^{-1} was tested (Fig. 4A, Strips #1-6). Only 50 µl of *Leptospira* suspension were used. This suggested that our strip demonstrated the positive result although only 50 leptospires were present.

Strips were also tested with other representative serovars. Suspensions of all leptospires used in this experiment were diluted to produce a final concentration of 10^3 leptospires ml^{-1} and

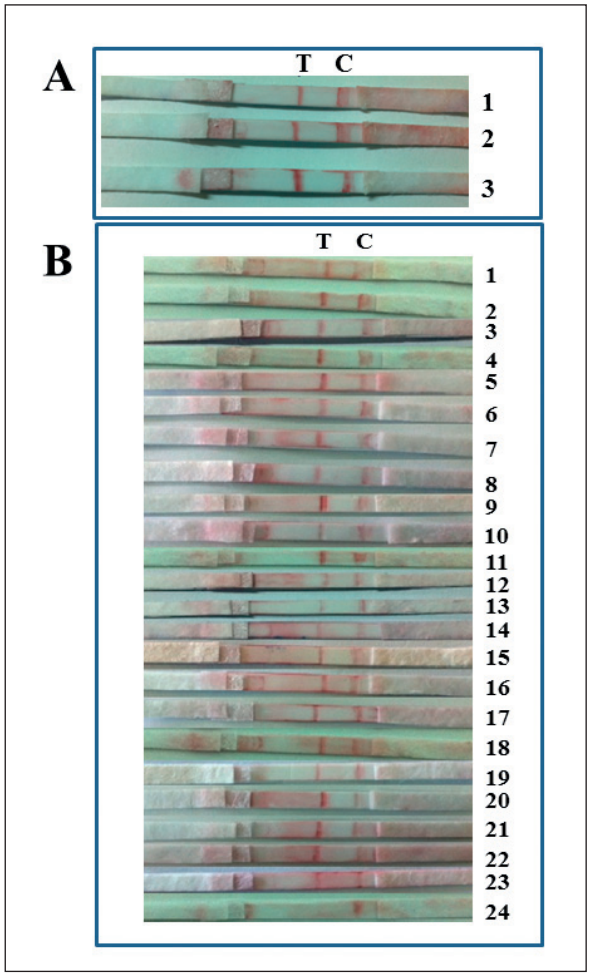

FIGURE 3 - *Leptospira* detection by immunochromatographic test strips. (A) Rabbit anti-LipL32 was coated on gold nanoparticles as described in Materials and Methods. Gold particles coupled with rabbit anti-LipL32 at final concentrations 10, 30 and 50 µg ml^{-1} were applied on the conjugate pad of Strips # 1, 2 and 3, respectively. All strips were tested with 50 µl of $10^8 ml^{-1}$ *Leptospira interrogans* serovar Bratislava. (B) Strips were tested with 23 pathogenic and a non-pathogenic *Leptospira*. Strips # 1-23 were tested with pathogenic *Leptospira* serovars Bratislava, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum, Sarmin, Sejroe, Shermani and Tarassovi, respectively. Strip # 24 was tested with *Leptospira biflexa* serovar Patoc. T = test zone, C = control zone.

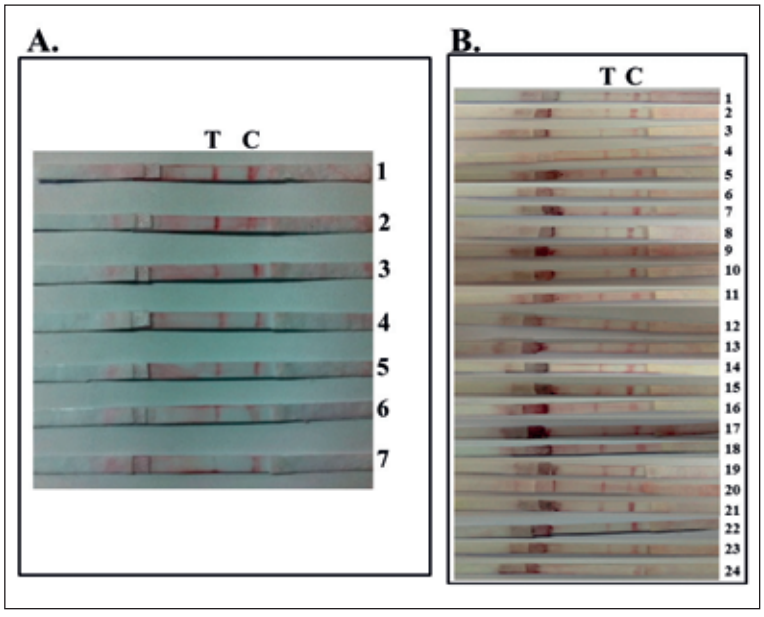

FIGURE 4 - *Sensitivity of Leptospira detection. (A) Strips were tested with various amounts of Leptospira interrogans serovar Bratislava. Strips # 1, 2, 3, 4, 5, 6 and 7 were tested with 50 ml of 10^8, 10^7, 10^6, 10^5, 10^4,10^3 and 10^2 ml^{-1} of Leptospira interrogans serovar Bratislava, respectively. (B) Strips were tested with 50 µl of 10^3 $ml^{-1}$of other representative serovars. Strips # 1-23 were tested with pathogenic Leptospira serovars Bratislava, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum, Sarmin, Sejroe, Shermani and Tarassovi, respectively. Strip # 24 was tested with non-pathogenic Leptospira (Leptospira biflexa serovar Patoc). T = test zone, C = control zone.*

50 µl of suspensions were used. As shown in Figure 4B, positive results were observed in every strip. These results confirm that the sensitivity of detection for all representative serovars is 50 leptospires or 10^3 ml^{-1}.

Sensitivity was also determined using urine or serum containing *Leptospira*. *Leptospira* serovar Bratislava were spiked into urine or serum from a normal individual at 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 leptospires ml^{-1}. As shown in Figure 5A and 5B, sensitivity of detection was the same (10^3 leptospires ml^{-1}) when strips were tested with urine or serum spiked with *Leptospira*. Cross-reactivity of test strips with other bacteria commonly isolated from urine or blood was tested. Suspensions of *Staphylococcus aureus*, *Streptococcus* group B, *Acinetobacter baumannii, Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Enterococcus faecium* were applied on test strips.

As shown in Figure 6 (Strips #2-9), no band was visualized on the test zone when these bacteria were tested.

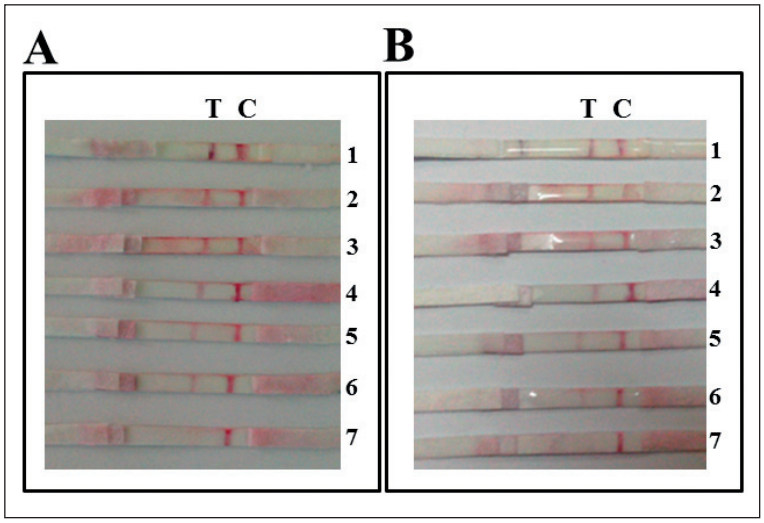

FIGURE 5 - *Sensitivity in urine and serum samples. Urine (A) or serum (B) from a normal control was spiked with various concentrations of Leptospira interrogans serovar Bratislava. Strips # 1, 2, 3, 4, 5, 6 and 7 were tested with 50 µl of 10^8, 10^7, 10^6, 10^5, 10^4,10^3 and 10^2 ml^{-1} of Leptospira interrogans serovar Bratislava, respectively. T = test zone, C = control zone.*

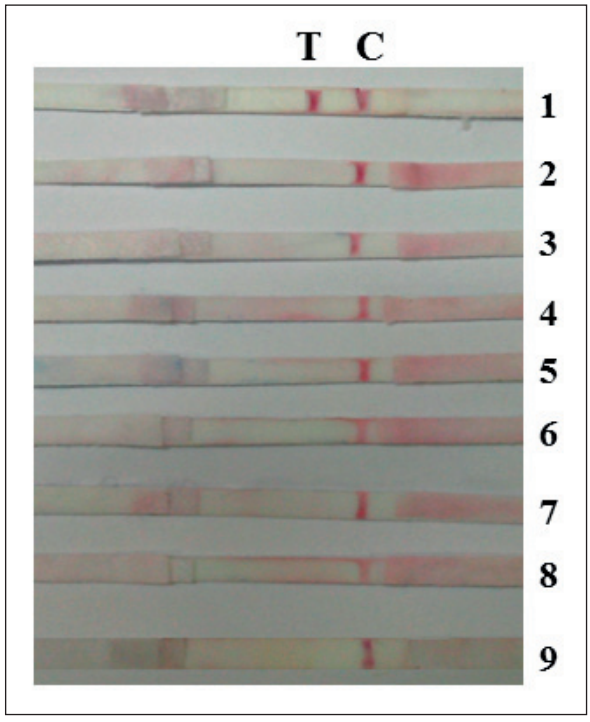

FIGURE 6 - *Determination of cross-reactivity with other bacteria. Strips # 1-8 were tested with suspensions of Leptospira interrogans serovar Bratislava, Staphylococcus aureus, Streptococcus group B, Acinetobacter baumannii, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Enterococcus faecalis, and Enterococcus faecium, respectively. T = test zone, C = control zone.*

DISCUSSION

Antibody detection has been commonly used for leptospirosis diagnosis. The diagnostic sensitivity of antibody detection is around 50% during the first week of infection because the amount of antibody present in blood during this time is still low. It has been shown that *Leptospira* DNA detection by PCR has higher sensitivity than antibody detection during the first week after infection (Flannery *et al.,* 2001; Vijayachari *et al.,* 2002; Fonseca Cde *et al.*, 2006).

LipL32 is an outer protein of *Leptospira* that has been widely studied as a vaccine candidate and as an antigen for diagnostic assays (Dey *et al.,* 2008; Feng *et al.,* 2009; Aviat *et al.,* 2010; Chalayon *et al.,* 2011; Sun *et al.,* 2011). In addition, LipL32 gene detection by PCR or real-time PCR has been reported (Levett *et al.,* 2005; Fernandes *et al.*, 2008; Lee *et al.*, 2011). We demonstrated that the immunochromatographic strips using gold particles coupled with anti-LipL32 could detect all pathogenic *Leptospira* tested in this study. The sensitivity of *Leptospira* detection was 10^3 leptospires ml^{-1} in both urine and serum. These data suggested that the strips could be further developed for detection of *Leptospira* in both urine and serum samples.

It has been shown that *Leptospira* are excreted in urine and can be detected before the eighth day of infection (Bal *et al.,* 1994). We demonstrated that the test strip could detect *Leptospira* present in urine. Detection of urine *Leptospira* by PCR was also reported (Fernandes *et al*. 2008; Lee *et al*. 2011). Urine spiked with *Leptospira* was used in these studies and the sensitivity of detection by PCR was around $10^2 ml^{-1}$. The sensitivity of our strip test was slightly lower than that of PCR. However, it has been shown that the average number of leptospires in urine of leptospirosis patients is 2.5 x $10^3 ml^{-1}$ and the critical threshold for vital diagnosis of leptospirosis patients is $10^4 ml^{-1}$of blood (Truccolo, *et al.*, 2001). The immunochromatographic strip test is much easier to perform than PCR and the result could be observed within five minutes.

In summary, we have demonstrated that an immunochromatographic assay targeting LipL32 is another promising tool for *Leptospira* detection. Additional data from further testing this assay with clinical samples will be collected to demonstrate the predictive value for disease diagnosis.

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REFERENCES

Ahmed A., Engelberts M.F., Boer K.R., Ahmed N., Hartskeerl R.A. (2009). Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS One.* **18**, e7093.

Appassakij H., Silpapojakul K., Wansit R., Woodtayakorn J. (1995). Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. *Am. J. Trop. Med. Hyg.* **52**, 340-343.

Aviat F., Rochereau-Roulet S., Branger C., Estavoyer J.M., Chatrenet B., Orsonneau J.L., et al. (2010). Synthetic peptide issued from Hap1/LipL32 for new early serodiagnosis of human leptospirosis. *Comp. Immunol. Microbiol. Infect. Dis.* **33**, 375-387.

Bal A.E., Gravekamp C., Hartskeerl R.A., De Meza-BrewsterJ., Korver H., Terpstra W.J. (1994). Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. *J. Clin. Microbiol.* **32**, 1894-1898.

Bharti A.R., Nally J.E., RicaldiJ.N., Matthias M.A., Diaz M.M., Lovett M.A. et al. (2003). Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* **3**, 757-771.

Boonyod D., Poovorawan Y., Bhattarakosol P.,Chirathaworn C. (2005). LipL32, an outer membrane protein of *Leptospira*, as an antigen in a dipstick assay for diagnosis of leptospirosis. *Asian Pac. J. Allergy Immunol.* **23**, 133-141.

Bourhy P., Bremont S., Zinini F., Giry C., Picardeau M. (2011). Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. *J. Clin. Microbiol.* **49**, 2154-2160.

Chalayon P., Chanket P., Boonchawalit T., Chattanadee S., Srimanote P., Kalambaheti, T. (2011). Leptospirosis serodiagnosis by ELISA based on recombinant outer membrane protein. *Trans. R. Soc. Trop. Med. Hyg.* **105**, 289-297.

Chirathaworn C., Kaewopas Y., Poovorawan Y., Suwancharoen D. (2007). Comparison of a slide agglutination test, LeptoTek Dri-Dot, and IgM-ELISA with microscopic agglutination test for *Leptospira* antibody detection. *Southeast Asian J.Trop. Med. Public Health.* **38**, 1111-1114.

Chirathaworn C., Chantaramalai T., Sereemaspun A., Kongthong N., Suwancharoen D. (2011). Detection of *Leptospira* in urine using anti-*Leptospira*-coated gold nanoparticles. *Comp. Immunol. Microbiol. Infect. Dis.* **34**, 31-34.

Cullen P.A., Cordwell S.J., Bulach D.M., Haake D.A., Adler B. (2002). Global analysis of outer membrane proteins from *Leptospira* interrogans serovar Lai. *Infect. Immun*. **70**, 2311-2318.

Cullen P.A., Xu X., Matsunaga J., Sanchez Y., Ko A.I., Haake D.A., Adler B. (2005). Surfaceome of *Leptospira* spp. *Infect. Immun.***73**, 4853-4863.

Dey S., Mohan C.M., Ramadass P., Nachimuthu K. (2008). Diagnosis of leptospirosis by recombinant antigen based single serum dilution ELISA. *Indian J.Med. Res.* **128**, 172-177.

Feng C.Y., Li Q.T, Zhang X.Y., Dong K., Hu B.Y., Guo X.K. (2009). Immune strategies using single-component LipL32 and multi-component recombinant LipL32-41-OmpL1 vaccines against *Leptospira*. *Braz. J. Med. Biol. Res*. **42**, 796-803.

Fernandes C.P., Seixas F.K., Coutinho M.L., Vasconcellos F.A., Moreira A.N., Conceição F.R., Dellagostin O.A., Aleixo J.A. (2008). An immunomagnetic separation-PCR method for detection of pathogenic Leptospira in biological fluids. *Hybridoma.* **27**, 381-386.

Flannery B., Costa D., Carvalho F.P., Guerreiro H., Matsunaga J., Da Silva E.D., et al. (2001). Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *J. Clin. Microbiol.* **39**, 3303-3310.

Fonseca CdeA., Teixeira M.M., Romero E.C., Tengan F.M., Silva M.V., Shikanai-Yasuda M.A. (2006). *Leptospira* DNA detection for the diagnosis of human leptospirosis. *J. Infect.* **52**, 15-22.

Haake D.A., Chao G., Zuerner R.L., Barnett J.K., Barnett D., Mazel M., et al. (2000). The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect. Immun*. **68**, 2276-2285.

Lee S.V., Tai E.S., Mutalib A.R., Khairani-Bejo S., Bahaman A.R. (2011). Rapid detection of pathogenic leptospires by lyophilized reagent-based Polymerase Chain Reaction. *Tropical Biomedicine.* **28**, 497-505.

Levett P.N., Morey R.E., Galloway R.L., Turner D.E., Steigerwalt A.G., Mayer L.W. (2005). Detection of pathogenic leptospires by real-time quantitative PCR. *J. Med. Microbiol.* **54**, 45-49.

Shekatkar S., Acharya N.S., Harish B.N., Parija S.C. (2010). Comparison of an in-house latex agglutination test with IgM ELISA and MAT in the diagnosis of leptospirosis. *Indian J. Med. Microbiol.* **28**, 238-240.

Silpasakorn S., Waywa D., Hoontrakul S., Suttinont C., Losuwanaluk K., Suputtamongkol Y. (2011). Performance of *Leptospira* immunoglobulin M ELISA and rapid immunoglobulin G immunochromatographic assays for the diagnosis of leptospirosis. *J. Med. Assoc. Thai.* **94**, (Suppl. 1), S203-206.

Sun A., Wang Y., Du P., Wu S., Yan, J. (2011). A sensitive and specific IgM-ELISA for the serological diagnosis of human leptospirosis using a rLipL32/1-LipL21-OmpL1/2 fusion protein. *Biomed. Environ. Sci.* **24**, 291-299.

Truccolo J., Serais O., Merien F., Perolat P. (2001). Following the course of human leptospirosis: evidence of a critical threshold for the vital prognosis using a quantitative PCR assay. *FEMS Microbiology Letters.* **204**, 317-321.

Vijayachari P., Sugunan A.P., Sehgal S.C. (2002). Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis. *Epidemiol. Infect.* **129**, 617-621.