Iranian Journal of Microbiology
Volume 6 Number 3 (June 2014) 184-189

Evaluation of New ELISA based on rLsa63 – rLipL32 antigens for serodiagnosis of Human Leptospirosis

Safar Ali Alizadeh[1], Gholamreza Abdolahpour[2], Mohammad Reza Pourmand[1], Taghi Naserpour[3, 4], Reza Najafipour[4, 5], Seyyed Saeed Eshraghi[1*]

[1]Department of Pathobiology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran. [2]Faculty of Veterinary Medicine, University of Tehran. [3]Department of Microbiology, Qazvin University of Medical Sciences, Qazvin, Iran. [4]Cell and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran. [5]Department of Biochemistry, Qazvin University of Medical Sciences, Qazvin, Iran.

Received: September 2013, **Accepted:** November 2013.

ABSTRACT

Background and objectives: Timely diagnosis of leptospirosis is essential for an effective treatment. Large diversity of clinical symptoms has led leptospirosis diagnosis difficult. Researchers have conducted many tests with wide-range of sensitivity and specificity to achieve novel diagnostic procedures which have higher sensitivity and specificity compared with previous tests and which are more reliable and available to public laboratories. This study aimed to introduce Lsa63 and LipL32 proteins-based ELISA tests with more sensitivity, specificity, accuracy and convenience for public laboratories.
Materials and Methods: Recombinant forms of Lsa63 and LipL32 proteins were first generated. After coating these proteins, IgM and IgG ELISA tests were performed. 220 patients with suspicion of leptospirosis infection were selected for serum collection. The sera tests were carried out using MAT, IgM and IgG ELISA tests. In order to assess the performance of ELISA, the results of this test were compared with MAT.
Results: 30% of serum samples (n=65) in MAT were positive for leptospirosis infection, while ELISA tests including rLipL32- rLsa63-IgM and rLipL32-rLsa63-IgG showed 40.45% (n=89) and 38.63% (n=80) positive reaction respectively.
Conclusion: Our results demonstrated that new ELISA tests based on mixing LipL32 and Lsa63 proteins, a novel mixture of recombinant antigens, are valuable to detect specific antibodies against pathogenic *Leptospira* in human serum and could be considered as helpful techniques in leptospirosis diagnosis.

Keywords: Lsa63 protein; LipL32 protein; ELISA; *Leptospira*; leptospirosis

INTRODUCTION

Leptospirosis is known as a zoonotic infection throughout the world. Humans always acquire leptospirosis through a direct or an indirect contact with infected animals, soil and surface water contaminated with *Leptospira* excreted from animals' urine (1-3). Clinical manifestations of leptospirosis vary from mild to severe. Fever, myalgia, headache, malaise, intense jaundice and bleeding are its main symptoms. The severest form of this infection named Weil's disease, causes pulmonary hemorrhage syndrome and liver or renal failure and consequently results in death. The major concern with leptospirosis is the large diversity of clinical symptoms which makes the diagnosis difficult and requires various laboratory tests (4-6). At the present time, techniques commonly used in laboratories for leptospirosis diagnosis are bacteriological culture (bacterial culture seems

*Corresponding author: Seyyed Saeed Eshraghi PhD
Address: Department of Pathobiology, Tehran University of Medical Sciences, PourSina St., Tehran, Iran
Tel: +98-21-88994823
Fax: +98-21-88954913
E-mail: eshraghs@tums.ac.ir

Table 1. The designed primers for amplifying *LipL32* and *Lsa63* protein genes by PCR.

Name	Primer sequence	Restriction enzyme site
LipL32-F	5′- CGCGCGGCAGCCATATGTGTGGTGCTTTCGGTGGTC -3′	*NdeI*
LipL32-R	5′- GGTGGTGGTGCTCGAGCTTAGTCGCGTCAGAAGCAG -3′	*XhoI*
Lsa63-F	5′- CGCGCGGCAGCCATATGGAAAGTTCTAAACTCGGAG -3′	*NdeI*
Lsa63-R	5′- GGTGGTGGTGCTCGAGAATCAGTTTTAGATCGGC -3′	*XhoI*

to be a better choice), microscopic agglutination test (MAT), serological tests (ELISA, IFA and agglutination based tests) and molecular tests such as PCR-based tests (2-3). These methods have several problems for a specific and sensitive leptospirosis diagnosis. The inconclusiveness of previous tests has led researchers to develop more definitive tests with higher sensitivity and specificity which could be readily available to public laboratories (6-8).

In this study we investigated two *Leptospira* antigens (Lsa63 and LipL32) which have been previously identified as adhesion proteins in the surface of pathogenic *Leptospira* serovars. These two antigens were selected as new serological markers (9-13). The aims of this study were to develop ELISA tests using recombinant Lsa63 antigen mixed with recombinant LipL32 antigen and to offer new tests for exploring the potential of these antigens in diagnosis of leptospirosis. This is the first study conducted to develop new ELISA tests based on the use of novel antigens for serodiagnosis of human leptospirosis. We produced the recombinant form of Lsa63 and Lipl32 antigens and developed two ELISA tests based on this novel mixture of antigens.

MATERIALS AND METHODS

Serum samples (n=220) were obtained from patients suspected of leptospirosis. The patients had jaundice, fever, headache, nausea and myalgia symptoms during their visits to local hospitals (Guilan University of Medical Sciences hospitals). The sera were divided into three aliquot parts and stored at -20°C until analysis. All serum samples were tested by MAT method as a golden test for serodiagnosis of leptospirosis.

Leptospira interrogans serovars namely Grippotyphosa, Pomona, Icterohaemorrhagiae, Canicola, Hardjo and Ballum were obtained from Leptospira Research Laboratory of Tehran University (http://www.leptolab.ut.ac.ir). These serovars are predominantly present in Guilan provinces of Iran where we collected our samples (14). These bacteria were maintained throughout the study for MAT performance. The MAT was done according to standard method using the panel of six references *Leptospira interrogans* serovars mentioned above (15). The 6 live *Leptospira* serovars were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) media for performing MAT on patient's sera. Serum specimens serially diluted in 96 well microtiter plates (flat bottomed) and then suspended live *Leptospira* cells were added to each well. The plates were examined for microagglutination reaction by dark field microscope after two hours incubation at 37°C. The reactions with maximum dilution of serum that agglutinated at least 50% of the cells for each serovars were marked as positive. Titer of each serum was measured. A positive MAT was considered at titer of ≥100 for single samples.

LipL32 and *Lsa63* genes were amplified from the genomic DNA of *L. interrogans* serovar Copenhageni by PCR with specific primers without the signal peptides (Table 1). The primers were designed by clontech software. (http://www.clontech.com/US/Products/Cloning_and_Competent_Cells/Selection_Guides/Online_In-Fusion_Tools)

The derived amplicons from each gene that encodes LipL32 and Lsa63 were cloned directly into *pET28a (+)* vector at *NdeI* and *XhoI* restriction sites by Clontech cloning kit (Clontech, USA). The inserted DNA combined with 6His tag at the 5′ end. The accuracy of cloning was verified by DNA sequencing of recombinant plasmids. The recombinant plasmids of each gene were then transformed into *E. coli* BL-21 (DE3) strain as expressing host. A single colony of *E. coli* BL21 (DE3) containing recombinant plasmids *pET28a (+)-LipL32* or *pET28a (+)-Lsa63* were selected and grown at 37°C on 5 ml Luria-Bertani broth (LB) containing kanamycin for overnight to inoculate 500 ml of LB broth containing kanamycin.

These two recombinant clones were induced by isopropyl-D-thiogalactopyranoside (IPTG) in a final concentration of 1mM. After five hours of induction, the bacterial cell pellet was harvested by centrifugation at 12000 rpm for 15 min and gently lysed by ultrasonication. The expressed proteins were analyzed by 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then measured by using Bradford biochemical method. Lysate of each protein was loaded into a Ni-NTA column [Qiagen; USA] to extract the N-terminal 6His tagged fused recombinant LipL32 and Lsa63 proteins under native conditions by gradient concentration of imidazole ranged from 150 to 350 mM. The first two ml of purified fractions containing the rLipL32 or rLsa63 proteins were collected and dialyzed in 0.01M PBS pH 7.2 overnight to remove the imidazole. Using SDS-PAGE, purity of extracted proteins were determined and then concentrated by freeze-dried lyophilizer.

Western blotting was done according to standard protocol using the anti-His-tag antibody. The extracted rLipL32 and rLsa63 were transferred from the SDS-PAGE separation gel to a nitrocellulose membrane (Dassel, Germany) by Biorad system. The blotting membranes were blocked with Phosphate-buffered saline (PBS pH 7.5) containing 3% bovine serum albumin (BSA) for one hour. The membranes were washed five times with PBS containing 0.05% Tween 20 (PBST). The blocked papers were incubated in a suitable dilution of Goat anti-rabbit IgG-Conjugated to horseradish peroxidase at 4°C for overnight. Finally, these membranes were then washed five times with TBST. Diaminobenzidine solution (DAB; Sigma, USA) was added to the papers for developing reactions.

A 1:1 dilution mixture of purified rLipL32 and rLsa63 was prepared in 0.06 M carbonate bicarbonate buffer pH 9.6 at a concentration and diluted to 1 ng/µl. Each well of an ELISA plate (MaxiSorp, Nunc) was loaded with 50 µl per well of the mixture proteins and incubated in a humid chamber at 4°C overnight. For removing unbound antigens, the coating plates were washed four times with 0.05% Tween phosphate buffered saline pH 7.4 (PBST) and all wells were blocked with 300 µl of 5% skim milk at 37°C for two hours. Excess blocking buffer was washed off five times with PBST. The best concentration of the antigens, antibodies and conjugate were determined by checkerboard plates (16). The biggest difference between optical density of positive and negative samples in ELISA were obtained by coating 50 µl (1 ng/µl) of rLipL32 and rLsa63 antigen, 1/50 dilution of serum and 1/5000 dilution of horse radish peroxidase (HRP) conjugate. Serum samples diluted 1:50 in diluent buffer (0.2% gelatin + 0.2% BSA in PBS) and was loaded 50 µl to ELISA plate wells. The plates were incubated at 37°C for one hour. After washing the plates, 50 µl anti-Human IgG or IgM conjugated to HRP (1:5000) were added to each wells as the second antibody to evaluate IgG or IgM level against *Leptospira* spp within sera from leptospirosis patients. The plates were washed five more times and then added 100 µl TMB substrate solution to the wells. Reactions were left to react for 15 minutes at room temperature and dark place. Finally, 100 µl stop solution (2 M H2SO4) was added to each wells. The absorbance value of wells was read using an ELISA reader at a wavelength of 405 nm. For determination of cut-off value, the mean cut-off point was calculated at OD450 + 3SD by obtaining the mean value of OD from the ELISA performed on 50 MAT negative control sera.

Statistical analysis, With 95% confidence interval, the accuracy, sensitivity, specificity and likelihood ratios for the ELISAs based on rLipL32 + rLsa63 were calculated in compared to the MAT assay as a golden test. All analyses were done using the user-written modules Diagt in stata software (release 10; StataCorp LP, College station, TX, USA).

RESULTS

The genes encoding the LipL32 and Lsa63 protein were cloned successfully into *pET28a(+)* expression vector by clontech system. The cloned plasmids [*pET28a (+)-LipL32, pET28a (+)-Lsa63*] were checked by colony PCR then performed double digestion by *NdeI* and *XhoI*. The digestion product was electrophoresed on 8 g/L agarose gel. The results showed that the recombinant plasmid contained the expected genes. The results of plasmid sequencing proved that the inserted sequences of *LipL32* and *Lsa63* into the vector were correct.

The recombinant clones highly expressed objective genes by IPTG and produced rLipL32 or rLsa63 proteins was purified in native condition. The analysis of SDS–PAGE results indicated that 32 kDa and 63 kDa proteins high expressed clearly which were

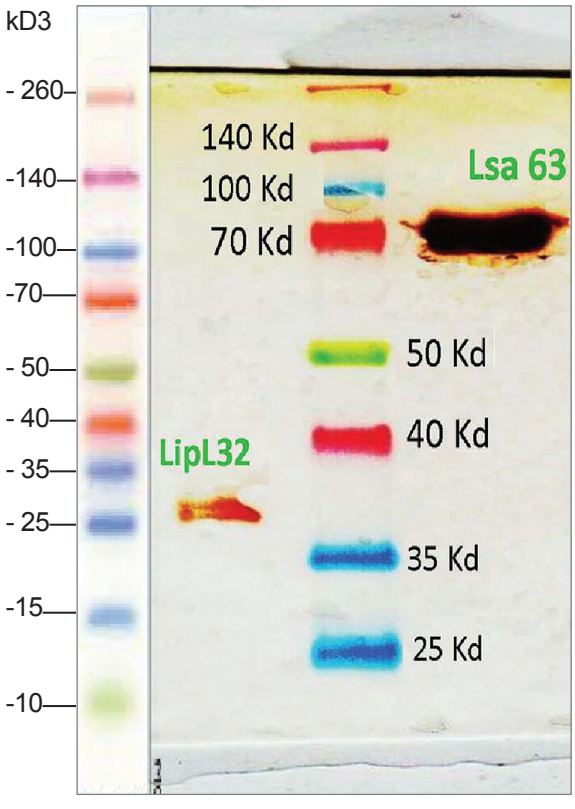

Fig 1. western blot of rLsa63 and rLipL32 proteins (Left to Right: Line 1 LipL32, Line 2 protein size marker, Line 3 Lsa63)

related to objective proteins. Recombinant LipL32 and Lsa63 proteins were evaluated by anti-6His tag antibody (Sigma, USA) in Western blot which the proteins appeared clearly visible bands 32 and 63 kDa highly expressed those were related to predicted proteins. (Fig. 1)

Cut off value for rLipL32-rLsa63-IgM-ELISA and rLipL32-rLsa63-IgG-ELISA were measured. Obtained Cut-off points were 0.45 and 0.42 for IgM and IgG of our ELISAs, respectively

Among 220 serum samples were obtained from patients who suspected of having leptospirosis, 65 (30%) were positive according to the MAT assay that considered positive specimens and 155 (70%) were negative. The rLipL32-rLsa63-IgM-ELISA and rLipL32-rLsa63-IgG-ELISA showed positive reaction 40.45% (n=89) and 38.63% (n=80), respectively. In compared to MAT, the rLipL32-rLsa63-IgM-ELISA had a 96.62%, a 83.23%, a 70.78% and a 98.47% sensitivity, specificity, PPV and NPV, respectively, and also rLipL32-rLsa63-IgG-ELISA had a 92.31%, a 83.87 %, a 70.59% and a 96.30% sensitivity, specificity, PPV and NPV, respectively. The summarized results have been shown in table 2.

DISCUSSION

Until now, the golden test for diagnosis of Leptospiral infections is MAT (3-4) but this test is not effective during early phases of the illness (2). To overcome this problem, different serological tests using single Leptospiral recombinant outer membrane proteins (OMP) such as Lipl32, LipL21, OmpL1/2, Lipl41 and rLoa22 and others have been established (6, 10, 17-20) Up to now, many researchers developed different ELISAs using the single recombinant Leptospiral surface proteins as the antigens (21-22), for example; Bomfim developed an ELISA by using single recombinant LipL32 for diagnosis of canine leptospirosis (9), Senthilkumar established an ELISA based on rLipL42 protein for canine leptospirosis diagnose and reported a sensitivity of 83.33% and specificity of 93.07% for it (16), Srimanote made an ELISA tests (IgM and IgG) with recombinant ligA (6), Dey developed an ELISA with the recombinant

Table 2. Test performances of the LipL32-Lsa63-ELISAs tests, when compared to the MAT

Test performances	IgM-Lsa63 ELISA	IgG-Lsa63 ELISA
Sensitivity (%) (95% CI)	96.62 (89.46 – 99.15)	92.31 (85.83 – 98.79)
Specificity (%) (95% CI)	83.23 (76.56 – 88.29)	83.87 (78.08 – 89.66)
Positive predictive value (%) (95% CI)	70.78 (60.19-79.9)	70.59 (60.90 – 80.27)
Negative predictive value (%) (95% CI)	98.47 (94.59- 99.81)	96.30 (93.11 – 99.48)
Positive likelihood ratio (95% CI)	5.778 (4.058 - 8.227)	5.723 (3.97 – 8.25)
Negative likelihood ratio (95% CI)	0.037 (0.009 - 0.145)	0.0917 (0.0394 – 0.2135)
Test accuracy (%) (95% CI)	87.27 (82.87 – 91.68)	86.36 (81.83 – 90.90)

LipL32 antigen for detection of anti-Leptospiral antibodies in human serum samples and reported an acceptable sensitivity and specificity for their tests (23), Oliveira developed ELISA based on MPL17 and MPL21 antigens that have specificity 95.5% and 80.6%, respectively (19).

Nevertheless, these tests cannot convince scientists to use them in public laboratory because the accuracy, sensitivity and specificity are not suitable. Therefore researchers studied new recombinant antigens for establishing new tests. Several reports demonstrated that use of more antigens have been improving the accuracy of immunoassay tests (19, 24-25). Sun have developed ELISA tests by using three antigens including rLipL32/1, LipL21 and OmpL1/2 in fusion form. They reported the IgM ELISA based on this fusion antigen with 93.1% positive result (25).

In the present study, we established ELISAs with the novel combination of two Leptospiral recombinant surface antigens (rLipL32 and rLsa63). The antigens were previously proved to react with serum from patient's suspected Leptospira infections. LipL32 is the most present antigen on the surface of Leptospira and is highly common among pathogenic Leptospira strains (9, 11). This protein is highly expressed during leptospirosis and recognized through the humoral immune reaction in patients (11-12, 25). Furthermore, previous study by Vieira also demonstrated that Lsa63 protein appears on surface of pathogenic Leptospira acts as an adhesion (13).

The immune system will recognize cell surface antigens while initial contact of pathogenic Leptospira occur, and produce antibodies against them. Since the antibodies generated against the surface antigens of the bacteria, recombinant form of these antigens may supply the best candidates for establishing diagnostic tests (18). These studies demonstrated that LipL32 and Lsa63 are unique to pathogenic Leptospires and that the antibodies related to these antigens were detected exclusively in Leptospira infected host sera (9, 11-13). These facts suggested that a mixture of recombinant form of LipL32 and Lsa63 antigens may be suitable to identify Leptospiral infection. Our special interest was to explore potential of LipL32 + Lsa63 mixture as an antigen in diagnosis of leptospirosis.

To our knowledge, this is a novel mixture that is used to develop ELISA tests for diagnosis of human leptospirosis with a defined set of sensitivity, specificity and accuracy in comparison to MAT as golden test. For this purpose, two ELISAs tests were generated by using the mixture for detection of specific IgM and IgG antibodies for pathogenic Leptospira serovars and then the serum samples assayed by them.

Our results showed that the rLipL32+Lsa63-IgM-ELISA and rLipL32+Lsa63-IgG-ELISA had high sensitivity (96.62% and 92.31% for 1:50 dilutions, respectively) recognizing specific antibodies directed to Leptospiral antigens. In contrast MAT using 65 human sera showed MAT titers ≥100 when the six serovars were tested. In this study, specificity of rLipL32+Lsa63-IgM-ELISA was 83.23 % and rLipL32+Lsa63-IgG-ELISA was 83.87% in comparative to the MAT when 155 MAT-negative sera were used. Our findings indicated that rLipL32+rLsa63 mixture is very immunogenic and when they are used together as a coated antigen could efficiently raise the detection accuracy, sensitivity and specificity and could thus be useful tool to develop a confirmative serological test.

Although we found that the LipL32 + Lsa63-ELISAs show good accuracy, specificity and sensitivity for diagnosis of human leptospirosis, but more confirmation of the tests in other populations from another area with other serovars prevalence is needed to validate of the present findings. Then there is a necessity for additional studies with a greater number of different patient sera from different region to evaluate its accuracy and making it a practical test for development of a serological kit for diagnosing of Leptospiral infections.

In conclusion, our findings suggested that (rLsa63 + rLipL32)-ELISAs is a specific, sensitive and practical test for the detection of specific antibodies against pathogenic Leptospira. In addition, the results of our ELISAs were obtained using a single sample (1/50 diluted serum), we suggest future studies use more recombinant antigens and also obtaining two serum samples each one or two weeks apart could further help to validate our results.

ACKNOWLEDGMENTS

This work was supported by the deputy of research of Tehran University of medical sciences. The authors would like to thank Mehdi Riazi for Leptospiral strains, and thank Dariush Ilghari for kindly technical help.

REFERENCES

1. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 2003; 3: 757-771.
2. Adler B. de la Pena Moctezuma A. *Leptospira* and leptospirosis. *Vet Microbiol* 2010. 140(3-4): p. 287–296.
3. Ahmed A, Engelberts MF, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic *leptospira* species in clinical materials. *PLoS One* 2009; 4:7093.
4. Ooteman MC, Vago AR, Koury MC. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. *J Microbiol Methods* 2006; 65: 247-257.
5. Croda J, Ramos JG, Matsunaga J, Queiroz A, Homma A, Riley LW, Haake DA et al. Leptospira Immunoglobulin-Like proteins as a serodiagnostic marker for acute Leptospirosis. *J Clin Microbiol* 2007; 45:1528-1534.
6. Srimanote P, Wongdeethai N, Jieanampunkul P, Samonkiert S, Leepiyasakulchai C, Kalambaheti T, et al. Recombinant ligA for leptospirosis diagnosis and ligA among the *Leptospira* spp. clinical isolates. *J Microbiol Methods* 2008; 72: 73-81.
7. Watt G, Jongsakul K, Suttinont C. Possible scrub typhus coinfections in Thai agricultural workers hospitalized with leptospirosis. *Am J Trop Med Hyg* 2003; 68: 89-91.
8. Perwez K, Suman VB, Kumar NA, Hussain MA. Leptospirosis in Febrile Patients: Diagnosis by serology and polymerase chain reaction. *J Biol Life Sci* 2011; 2:1–10.
9. Bomfim, M.R., A. Ko, and M.C. Koury, Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. *Vet Microbiol* 2005; 109: 89-94.
10. Dey S, Mohan CM, Kumar TM, Ramadass P, Nainar AM, Nachimuthu K. Recombinant LipL32 antigen-based single serum dilution ELISA for detection of canine leptospirosis. *Vet Microbiol* 2004; 103: 99-106.
11. Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Morais ZM, Barbosa AS et al. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun* 2008; 76: 2642-2650.
12. Murray GL, Srikram A, Hoke DE, Wunder EA Jr, Henry R, Lo M, et al. Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. *Infect Immun* 2009; 77: 952-958.
13. Vieira ML, de Morais ZM, Gonçales AP, Romero EC, Vasconcellos SA, Nascimento AL. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect* 2010; 60: 52-64.
14. Ehsanollah S, Gholam RA. Detection of leptospiral antibodies by microscopic agglutination test in north-east of Iran. *Asian Pac J Trop Biomed* 2011; 1: 227-229.
15. Vijayachari P, Sugunan AP, Sehgal SC. Role of microscopic agglutination test (MAT) as a diagnostic tool during acute stage of leptospirosis in low and high endemic areas. *Indian J Med Res* 2001; 114: 99-106.
16. Senthilkumar TMA, Subathra , Ramadass P. Evaluation of recombinant leptospiral antigen LipL41 in enzymelinked immunosorbent assay and latex agglutination test for serodiagnosis of canine leptospirosis. *Veterinarski archiv* 2007; 77: 475-484.
17. Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, Da Silva ED, et al. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *J Clin Microbiol* 2001; 39: 3303-3310.
18. Palaniappan RU, Chang YF, Hassan F, McDonough SP, Pough M, Barr SC et al. Expression of leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. J *Med Microbiol* 2004; 53: 975-984.
19. Oliveira TR, Longhi MT, de Morais ZM, Romero EC, Blanco RM, Kirchgatter K, et al. Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. *Clin Vaccine Immunol* 2008; 15: 1715-22.
20. Chalayon P, Chanket P, Boonchawalit T, Chattanadee S, Srimanote P, Kalambaheti T.Leptospirosis serodiagnosis by ELISA based on recombinant outer membrane protein. *Trans R Soc Trop Med Hyg* 2011; 105: 289-297.
21. Okuda M, Sakai Y, Matsuuchi M, Oikawa T, Watanabe M, Itamoto K et al. Enzyme-linked immunosorbent assay for the detection of canine *Leptospira* antibodies using recombinant OmpL1 protein. *J Vet Med Sci* 2005; 67: 249-254.
22. Theodoridis D, Böhmer J, Homuth M, Strutzberg-Minder K. Development of a novel ELISA for serodiagnosis of Leptospirosis and additional detection of pathogenic *Leptospira* by polymerase chain reaction for veterinary routine diagnostics. *Rev Cubana Med Trop* 2005; 57: 49-50.
23. Dey S, Mohan CM, Ramadass P, Nachimuthu K.. Diagnosis of leptospirosis by recombinant antigen based single serum dilution ELISA. *Indian J Med Res* 2008; 128: 172-177.
24. Natarajaseenivasan K, Vijayachari P, Sugunan AP, Sharma S, Sehgal SC. Leptospiral proteins expressed during acute & convalescent phases of human leptospirosis. *Indian J Med Res* 2004; 120: 151-159.
25. Sun A, Wang Y, DU P, Wu S, Yan J. A sensitive and specific IgM-ELISA for the serological diagnosis of human leptospirosis using a rLipL32/1-LipL21-OmpL1/2 fusion protein. *Biomed Environ Sciences* 2011; 24:.291-299