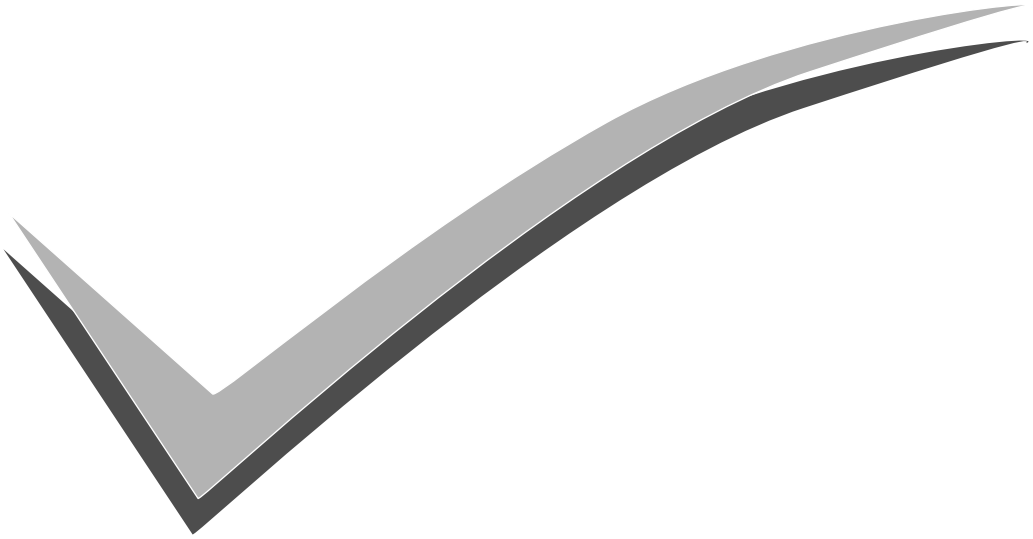
CE

ISO 13485:2016

Performance Evaluations



LEPTOCHECK®-WB

Rapid test for the detection of IgM antibodies to Leptospirosis

Performance Evaluations

CE

ISO 13485:2016

INDEX

S. No.	Name of the Publication	Pg Nos
1	International Journal of Research in Pharmaceutical and Biomedical Sciences, Vol. 4 (2) Apr– Jun 2013	412-416
2	National Journal of Medical ResearchVolume 2 Issue 1 Jan – March 2012	42-46
3	National Journal Of Community Medicine 2011 Volume 2 Issue 1	64-70
4	Journal of Clinical and Diagnostic Research. 2015 Feb, Vol-9(2)	21-24
5	www.aissl.org/ABSTRACTS-Basic%20Immunology.pdf	1/1
6	PLOS ONE | doi:10.1371/journal.pone.0129236 June 18, 2015	1-12
7	Journal of Infection and Public Health 12 (2019)	263–269
8	European Journal of Clinical Microbiology & Infectious Diseases, April 2022	1-7
9	PubMed,Wien Klin Wochensc, September 2015	1-7
10	BMC Infectious Diseases (2015) 15:319	1-5
11	Journal of Applied and Natural Science 6 (1): (2014)	12-13
12	Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 110 (4), June 2015	485-491
13	Indian Journal of Basic and Applied Medical Research; June 2015: Vol.-4, Issue- 3	8-11
14	IBMBB Abstract 17	1/1
15	Annals of Clinical Microbiology and Antimicrobials (2015) 14:2	1-8

Performance Evaluations

CE

ISO 13485:2016

OTHER EVALUATIONS

INDEX	
S. No.	**Name of the Evaluating Body**
16	Royal Tropical Institute (KIT) Amsterdam, June 2003
17	Leptospirosis Reference Laboratory, Amsterdam

Reference Articles on Google Books

Issues in Medical Microbiology, Mycology, Virology, and Molecular Medicine, Pg 26.

International Journal of Research in Pharmaceutical and Biomedical Sciences | ISSN: 2229-3701

Research Article

A Clinico Microbiological Study of Leptospirosis

H.Kar*,A. Urhekar,C.Pai (Bhat), A. Hodiwala (Bhesania) and M. Bhattacharjee

Department of Microbiology,MGM Medical College,Kamothe,Navi Mumbai,India.

ABSTRACT

Leptospirosis is an endemic and epidemic febrile disease caused by *Leptospiraicterohemorrhagiae*. It is recognized as an occupational hazard mainly in farmers, sewers and miners.The diagnosis of Leptospirosis poses a challenge & hence optimum techniques for the same are a must. This study has focused on comparing various methods for early laboratory diagnosis of Leptospirosis.50 clinically suspected cases of Leptospirosis were studied. Blood and urine samples were subjected to various tests like dark ground microscopy, Fontana's stain, culture (Fletcher's medium) and serology (Leptocheck for IgMantibodies and LeptoIgM ELISA) and other tests.The commonest clinical presentation in our study was of fever (100% cases), Headache (80 %) and Myalgia (13 %) and Icterus in 42 % cases. Among 50 clinically suspected cases of Leptospirosis, positive results were seen in 15 (30%) Leptocheck test, in 13 (26%) of IgMELISA, 03 (6%) of Culture and 5(10%) of Dark ground microscopy.Among all the tests for diagnosis maximum sensitivity was seen in the case of Leptocheck test. Culture, the gold standard is time taking and cumbersome and is difficult to adopt in routine diagnostics.

Keywords: Leptospirosis, Leptocheck test, Leptospiraicterohemorrhagiae, Fletcher's medium.

INTRODUCTION

Leptospirosisis an endemic and epidemic febriledisease. Ithas been recognized as an occupational hazard mainly in farmers, sewers, miners, caused by infection with pathogenic spirochete of genus *Leptospira*.The organisms are maintained in the nature by chronic renal infection of the carrier animals like rats which excrete the organism in their urine. Hence this study aims to find out the main presenting complaints of patients suspected of Leptospirosis by Faine's criteria, to correlate with age, sex and occupation of patients,to carry out various laboratory tests for diagnosis of Leptospirosis & to study the treatment and clinical outcome.

MATERIALS AND METHODS

The study was carried out at the department of Microbiology over a period of 2 years from 2008 to 2010.Sample collection: Total of 50 blood & urine samples from clinically suspected cases of leptospirosis were obtained from in-patient and outdoor patient. Urine was alkalinized by addition of sterile soda bicarb. For control, 25 blood samples were collected from healthy persons.

Laboratory tests performed:

1. Dark ground microscopy of centrifugal deposit of plasma and urine.
2. Fontana's staining of above centrifuged deposit.
3. Culture in semi solid Fletcher's medium with rabbit serum.
4. Rapid serological test with Leptocheck (IgM antibody)
5. IgM ELISA for Leptospirosis.
6. CBC, LFT, RFT and routine microscopy from haematology and biochemistry lab.

RESULTS AND DISCUSSION

A total of 50 samples of blood and urine were studied by Dark ground microscopy, Fontana's stain, Culture, Rapid test and ELISA test. Control blood samples were obtained from 25 healthy persons were studied by ELISA IgM test.Of the 50 patients we studied, 43 (86%) of the patients were male, while 7 (14%) were females (Ratio 6:1).CDC (Center for Disease Control and prevention, USA) studied distribution of leptospirosis by gender from year 2000-2006. Their ratio of male to female is about five times (5:1). High incidence of males due to their work in high potential infection areas like farm, sewage, mines.[1]

The maximum numbers of patients (64%) were from the age group of 25-50. This age group comprises of the occupationally active population. Similar results have been found in various other studies.[2,3]

Most of the patients were from the rural area and factors like water logging and improper sanitary conditions are mainly responsible for it.

There is sudden rise in the occurrence from July till October corresponding to the monsoon season.CDC data also clearly mentions that highest epidemic season for leptospirosis is related to the activity of the monsoon.[4]

Fever was the most common symptoms seen in 100% patients. This is consistent with the study conducted by Dey*et al*[5]Data published worldwide

shows the incidence of icteric manifestations ranging from 16% to 100%.[6] In our study, it was 66% and similar to the other study conducted in Mumbai by Dey*et al*[7]

Following significant laboratory findings were observed in leptocheck positive patients. Low platelet count 73.3%, high bilirubin level 80%, high SGOT and SGPT 62% and 66% respectively. High levels of BUN were observed in 80% cases. These findings suggests involvement and damage to liver and kidney.

Table no 5&6 is showing comparison of different laboratory test in the diagnosis of leptospirosis. Highest number of cases weredetected by the rapid test followed by IgM ELISA and culture. Most of the patients come to the hospital after a week of illness and after having consumed antibiotics. This also explains the low rate of detection of infection by culture, as these tests are usually positive early in the first week of illness. Chandrasekaran has demonstrated isolation rate by culture of 8.3% from the clinical samples, which is similar to our rate of 6.0%. [8]

Thus we consider that the sera that were positive by both Leptocheck as well as IgM ELISA could be of the *Leptospira* infected patient. In addition the positive results by these two assays are adequate for the diagnosis of leptospirosis in early stages and for initiating the specific anti Leptospiral treatment.

Thus to conclude, the study highlights the importance of emerging leptospirosis in and around Mumbai. Incidence of leptospirosis is often under reported due to the lack of clinical suspicion due to diverse manifestations. Rapid test like the Leptocheck is the method of choice for Rapid diagnosis. It should be supplemented with IgM ELISA and if possible MAT.

The MAT, which is the gold standard, is not available to all laboratories.

CONCLUSION

The common clinical presentations in our study are fever (100%), headache (80 %) and myalgia (13%). Icteric manifestations were seen in (42%) cases.Other clinical features encountered were acute renal failure (20%), ARDS (2%) Conjunctival suffusion (2%), Bleeding diathesis (5%), Anuria (20%) & CNS involvement was (4%).

86% of the patients were males and majority of them were in the age group 25-50. 63 % of the patients were from rural areas.There was a peak in the incidence of Leptospirosis from July to September i.e. during the monsoon period. Faine's clinical criteria for diagnosis were evaluated. They showed a sensitivity of 83.3% and specificity of 70.9%.

Culture which is considered gold standard for most of the diseases was positive in only 6.0% of clinically suspected cases. It is tedious and time consuming to perform.Lower isolation due to prior antibiotic treatment.

Rapid test (Lepto check) was positive in 15 patients (30% cases). These samples were subjected to test by panbioIgM ELISA test. ELISA test was positive in 13 patients. Thus the correlation was good between the two tests.

Comparing the two laboratory tests, the Rapid test(Lepto Check) gave the higher sensitivity (91.3%) as compared to ELISA. The control 25 healthy patients were tested by IgM ELISA, all were negative.

A combination of tests was also evaluated with a view to develop a diagnostic protocol. The combination of Rapid and IgM ELISA gave highest sensitivity. The disadvantage of the Rapid test which gave false positivity (13.3%) could be overcome by addition of an IgM ELISA which was more specific.

Table 1: Age distributionLeptospirosis (n=50)

	PAEDIATRICS No.(%)	ADULT No.(%)	TOTAL
Clinically suspected patients	03 (6%)	47 (94%)	50

Table 2: Sex distribution of clinically suspected patients ofLeptospirosis (n=50)

	MALES No. (%)	FEMALES No. (%)	TOTAL
Clinically supspected patients	43 (86%)	7 (14%)	50

Table 3: Clinical manifestations and correlation with leptocheck test. (n=50)

SYMPTOMS AND SIGNS SYMPTOMS	No.(%) Suspected Tested (50)	Leptospirosis Sero + Leptocheck (n=15)	Leptocheck Negative (n=35)
Fever with chills	50 (100%)	15(100%)	35(100%)
Head ache	15 (30%)	7(46.7%)	8(22.9%)
Vomiting	34(68%)	12(80%)	22(62.9%)
Myalgia	25(50%)	7(46.7%)	18(51.4%)
Haematemesis	1(2%)	1(6.7%)	00
Anuria	3(6%)	3(20.0%)	00
SIGNS Temp. >= 100° C	32(64%)	15(100%)	17(48.6%)
Subcunjuctival Suffusion	3(6%)	3(20.0%)	01(2.85%)
Icterus	15(30%)	10(66.7%)	5(14.3%)
Hepatomegally	12(24%)	7(46.7%)	5(14.3%)
Petechial Haemorrhage	1 (2%)	1(6.7%)	01(2.85%)
Hemoptysis (ARDS)	1 (2%)	1(6.7%)	00

Table 4: Routine laboratory parameters of *Leptospira* suspected Patients (n=50)

LABORATORY PARAMETERS	NORMS	Leptocheck Positive (n=15)	Leptocheck Negative (n= 35)
Low Hb % (< 11 gm%)	M 14 – 18 gm F 12 – 16 gm	1(6.7%)	1(2.9%)
High TLC (Leukocytosis) > 11000 /cu mm	5000 – 11000 /mm ^{3}	9(60.0%)	26(74.3%)
Low Platelet count < 1.5 lac /cu mm	1.5 – 4.0 lacs/mm ^{3}	11(73.3%)	1(2.9%)
High bilirubin > 1 mg/dl	0.3 – 1.0 mg/dl	12(80.0%)	13(37.1%)
High SGPT	0 – 35 IU/L	12(80.0%)	19(54.3%)
High SGOT	0 – 35 IU/L	12(80.0%)	21(60.0%)
High Alkaline Phosphatase	36 -141 IU/L	2(13.3%)	1(2.9%)
High BUN	10 – 20 mg/dl	12(80.0%)	17(48.6%)
High Serum creatinine	0.5 – 1.5 mg/dl	6(40.0%)	8(22.9%)
Urine R/M Alb/Hematuria/Pyuria		10(66.7%)	1(2.9%)

Table 5: Positive laboratory parameters of*Leptospira* suspected patients (n=50)

Blood DGM	Urine DGM(Suspected)	Culture
5(10%)	3(6%)	3(6%)

Table 6: Positive serological results in *Leptospira* suspectedpatients (n= 50)

IgM (Lepto Check)	ELISA
15(30%)	13 (26%)

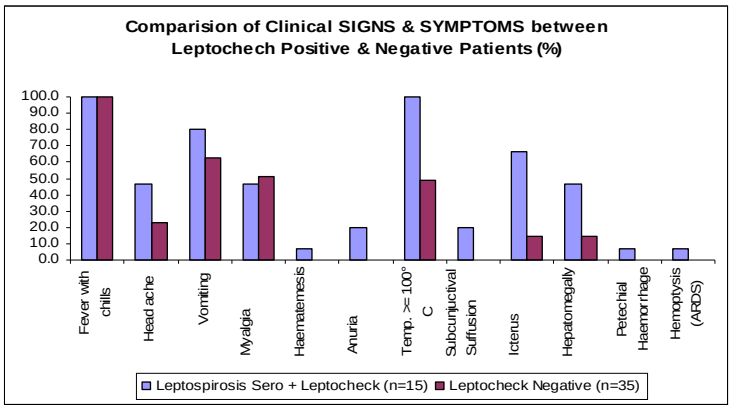


Fig. 1: Showing comparison of signs and symptoms between Leptocheck positive and negative patients

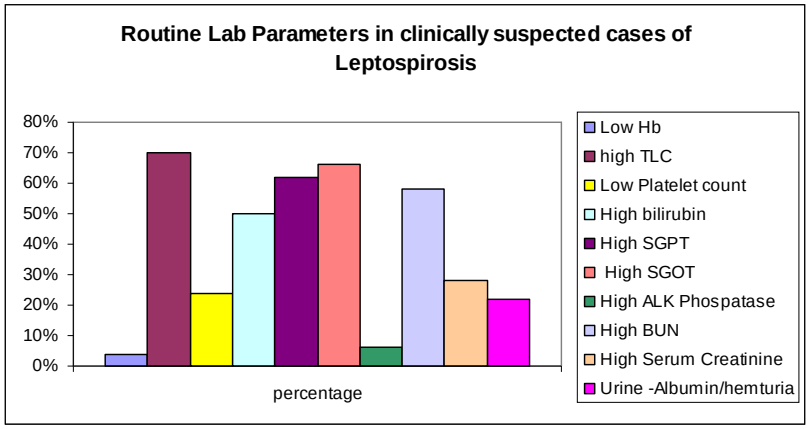


Fig. 2: Showing routine lab parameters in clinically suspected cases of Leptospirosis

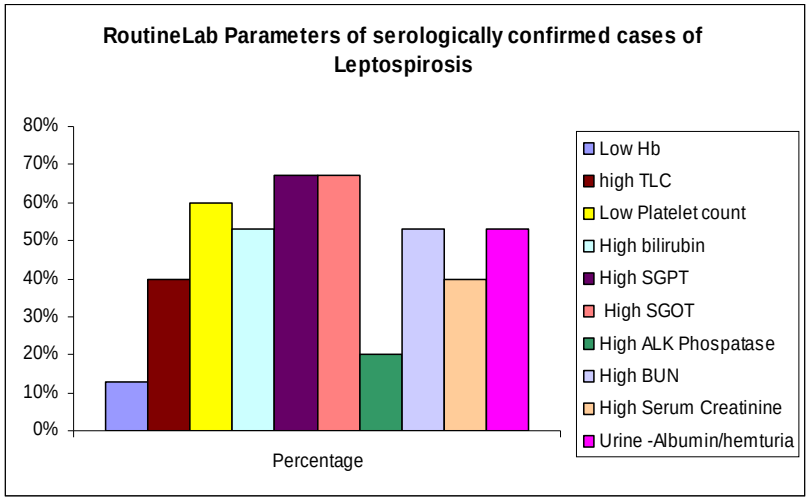


Fig. 3: Showing routine lab parameters of serologically confirmed cases of Leptospirosis

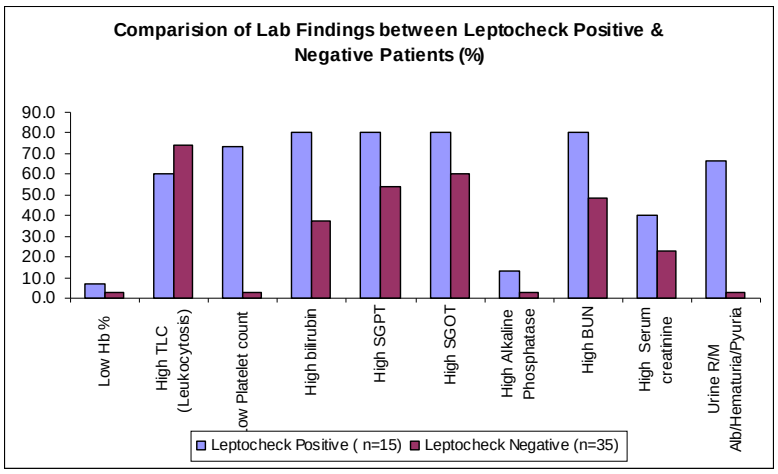


Fig. 4: Showing comparison of lab findings between Leptocheck Positive and Negative patients

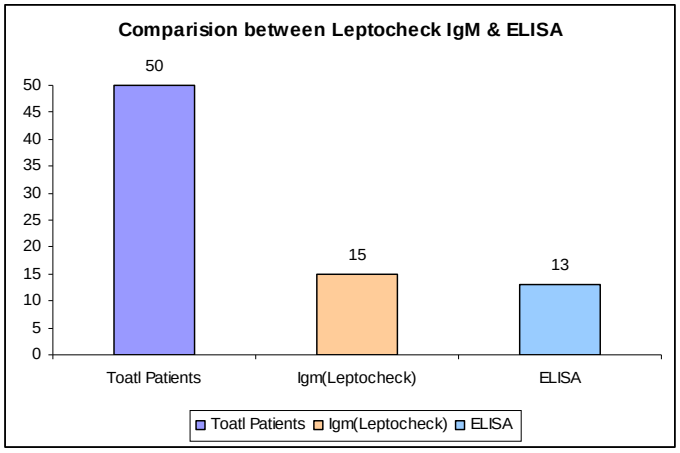


Fig. 5: Showing comparison between Leptocheck and IgM ELISA

REFERENCES

1. World Health Organization. Leptospirosis worldwide,1999. WklyEpidemiol Rec 1999; 74:237–42.
2. Angnani R, Pathak A A, Mishra M. Prevalence of leptospirosis in various risk groups. Indian J Med Microbiology 2003: 21;271-273
3. Chandrasekaran S. Review on human leptospirosis. 1999. Indian journal of Medical sciences; 53:291-297.
4. www.cdc.gov.tw/.../Health%20topics/Communicable%20Diseases%20&%20Prevention/2000-2006_epidemiology.pdf
5. De A, Varaiya A, Pujari A, Mathur M, Bhat M, Karande S, Yeolekar M E. An outbreak of leptospirosis in Mumbai. Indian Journal of Medical Microbiology;2002: 20 (3);153-155.
6. Eliana Dias Matos, Everaldo Costa, Edilson Sacramento, Anna Luiza Caymmi, César de Araújo Neto, Marcelo Barreto Lopes and Antonio Alberto Lopes.The Brazilian Journal of Infectious Diseases 2001: 5(2): 73-77.
7. Guidelines for prevention and control of leptospirosis. Zoonosis division 2006. National institute of communicable diseases.
8. Report of the Brainstorming Meeting on Leptospirosis Prevention and Control Mumbai, 16 – 17 February 2006.Joint Publication by: Office of WHORepresentative to India, New Delhi and regional medical research center (Indian Council of Medical Research).

ORIGINAL ARTICLE

OUTBREAK SURVEILLANCE REPORT ON PULMONARY LEPTOSPIROSIS AFTER A HEAVY FLOODS DURING 2006 IN SOUTH GUJARAT

Latika J Shah[1], Summaiya Mulla[2], Manish G Patel[3], Geeta Vaghela[3]

[1]Assistant Professor, [2]Professor & Head, [3]Associate Professor, Department of Microbiology, Government Medical College, Surat

Correspondence:
Dr. Latika Shah
51, Aagam Heritage, Near Someswara Enclave,
Opp.Landmark Honda, Vesu, Surat – 395 007
Email: drlatikanitin@yahoo.co.in

ABSTRACT

Background: During the heavy rainfall season in the Surat district of South Gujarat India, from July to October 2006 an outbreak of leptospirosis occurred.
Aim: This article reports the exposure of leptospirosis in this post flood outbreak. In total 1,258 patients of New Civil Hospital in Surat were included, based on their clinical signs and symptoms for leptospirosis. Severe pulmonary hemorrhages were observed in the imperative form in most cases encountered during this season.
Method: Laboratory investigation was carried out using rapid diagnostic tests like Leptocheck WB, Serion IgM ELISA and real-time PCR and they were evaluated for the outbreak investigation in comparison with the microscopic agglutination test (MAT)
Observation and Results: The predominant serovars encountered by the gold standard MAT were *autumnalis*(46%), *australis*(38%), *pyrogenes*(30%), *cynopteri*(20%), *icterohemorrhage*(8%) and g*rippotyphosa*(1.6%). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of rapid tests were analyzed, Leptocheck WB (91%, 78.4%, 83% & 88.3%), Serion IgM ELISA (92.2%, 89.4%, 90.3% & 91.6%) and Real time PCR (90.3%, 91.6%, 96.02% & 96.02%) using statistica (6.0). The incidence of the disease was greater during the month of August (41.41%) and September (52.94%) with a relative risk of 33.5 in Surat.
Conclusion: This implicates the impact of the heavy rainfall and flood as the cause for severe outbreak of leptospirosis among the urban population of Surat district. Frequently contaminated environmental exposures due to urbanization and industrialization were speculated as major cause for this severe epidemic during heavy floods, which entails preventive strategies and prompt treatment against leptospirosis under such outbreak circumstances.

Keywords: Leptospirosis, outbreak, MAT, Real time PCR, Leptocheck, IgMELISA

INTRODUCTION

Leptospirosis is a zoonotic disease having worldwide distribution and is caused by Genus *Leptospira*. The causative agent *Leptospira* is mainly transmited to humans through the environment or direct contact with urine from infected animals[1]. Infections with pathogenic *Leptospira* are increasingly recognized as a common cause of acute febrile illness in tropical environments[2]. The incidence of pulmonary involvement in Leptospirosis has been reported to be increasing and among 70% of the patients, alveolar hemorrhages dyspnea and hemoptysis are the predominant manifestations[3]. It is most common in tropical countries like Nicaragua [4, 5], India[6] and Thailand[7]. Pulmonary involvement in leptospirosis was first observed in India during outbreaks in Andaman Islands[8]. In Australia also pulmonary hemorrhage has been reported in patients with leptospirosis[9]. In past two decades, there is an increase in the number of cases of leptospiral pulmonary hemorrhages especially from Southeast Asia. This is mainly due to longer survival of *Leptospira* in environments with warm and humid conditions. Leptospirosis is a seasonal disease and the incidences mainly occur during the rainy season. The usual portal of entry is through abrasions or via the conjunctiva or intact skin after prolonged immersion in water [10,11]. Water-borne transmission has been documented in outbreak situations of Leptospirosis, usually after flooding. Apart from seasonal epidemics, the flood related outbreaks have increased the attentiveness of the epidemiologists to identify the cause and source of Leptospirosis.[12, 13]

Leptospirosis is a disease with protean manifestations, ranging from subclinical cases in the anicteric form to

the severe icteric form known as Weil's disease are characterized by a fulminant course with rapid onset of hepatic and renal failure and high mortality. Incubation period varies from 7 to 12 days but may range from 2 to 20 days. Leptospirosis classically presents as a biphasic illness. The first phase of the disease is commonly referred to as the septicemic phase. It is characterized by fever, headache, myalgia, conjuctival congestion and a host of non-specific features that may include mild cough, lymphadenopathy, rash, anorexia, nausea, and vomiting. This phase is followed by a brief febrile period of variable duration that, in turn, is followed by the immune phase of the illness.[2, 9] The common organs involved during this phase are the liver, lungs and kidneys. Both organ derangements are reversible.[14,15]

Leptospirosis diagnosis mainly rely on serological methods, Microscopic Agglutination Test (MAT) which remains useful for epidemiologic studies, identification of strains, assessment of the probable infecting serovar and confirmation of illness for public health surveillance. [16] In this report we discussed our experience of 2006 post flood Leptospirosis outbreak in Surat and the clinical presentation of the cases. The rapid diagnostic tests like Leptocheck WB, Serian IgM ELISA and real time PCR were evaluated in comparison with Microscopic Agglutination Test (MAT) during this severe disaster condition.

MATERIALS AND METHODS

Surveillance site

The City of Surat is located in the Southern part of Gujarat at 21° 15' N latitude and 72° 52' E longitude on the Southern bank of Tapti River, where the total population of Surat is approximately 4 million. During summer the temperatures range from 37.78°C to 44.44°C. The climate is pleasant during the monsoon season, while autumn is temperate. The winters are not very cold but the temperatures in January range from 10°C to 15.5°C. The average annual rainfall of the city has been 1143 mm. During August 2006 there was heavy rainfall all over India, but it was heavier in Madhya Pradesh state. The sudden release of a huge amount of water from the Ukai dam led to over 80 per cent of Surat going under water. More than 2 million people were trapped in their houses without food and drinking water for four days and four nights. The floods that ravaged Surat on 7th August left millions of people homeless and marooned thousands of animals. The rains disrupted communications, power and water supplies to the city. The transport system between Surat and other districts were cut off because of the raging waters from the Tapti river. As water receded in Surat the entire city was transformed into a garbage dump, with two feet of mud and muck on the streets. Hundreds of Leptospirosis cases were reported during the subsequent weeks which accounted for the large epidemic.

Patients and criteria used for clinical diagnosis

All the 1258 patients admitted, with clinical suspicion for Leptospirosis was included in the investigation. Among them 744 were males and 614 were of females. Investigations were carried out during the outbreak and observed that all patients had a high grade fever, headache and generalized body aches, associated with at least any one of the following sets of signs and symptoms. They included, according to criteria laid down by Indian Leptospirosis Society, a) jaundice, b) oliguria, c) cough, hemoptysis and breathlessness, d) neck stiffness with altered sensorium, and e) hemorrhagic tendencies including conjuctival suffusion and others.

Case confirmation by serological examination

As a part of the surveillance protocol, acute and convalescent- phase serum samples were obtained from suspected patients within 24 hours of admission. Among the cases, 675 paired sera were possible and they were collected in a mean interval of (> 14 days). Patients fulfilling any of the following criteria were considered as cases of leptospirosis: i) positive isolation of leptospires from blood or urine, ii) seroconversion or four fold titer in MAT for those with paired samples, iii) A titer of 1:80 or more with a positive IgM ELISA (titer of 1:80).

Serovar Specific microscopic agglutination test (MAT)

MAT was performed on the samples using eleven live leptospiral strains as antigens. The strains belonged to the serovars *australis* (JezBratislava), *autumnalis* (Bankinang) *ballum* (Mus127), *sejroe* (Hardjoprajitno), *grippotyphosa* (MoskvaV), *canicola* (HondUtrechIV), *hebdomadis* (Hebdomadis), *pomona* (Pomona), *patoc* (PatocI), *pyrogenes* (Perepelician), *icterohaemorrhagiae* (RGA). All the strains were obtained from Leptospira WHO Reference Centre, Port Blair and maintained with periodical subculture in Ellinghausen McCullough Johnson and Harris (EMJH) medium (Difco) at Department of Microbiolgoy, Government Medical College, Surat. The seven days old cultures having a concentration of 1-2x10^8 were used as antigen as per standard procedures.[17]

Rapid genus specific tests

Rapid genus specific tests like Leptocheck-WB (Zephyr Biomedicals, India) and Serion IgM EISA (Serion GmbH, Germany) were performed as per the manufactures instructions.

Real Time PCR assay

Total DNA from human serum (200 µl) was prepared using QIAamp DNA Mini Kits (QIAGEN, USA) according to the manufacturer's instructions. The primers and probes were designed from alignments of available *Leptospira spp. LipL41* sequences obtained from the GenBank nucleotide sequence database. The program used was Primer Express™ (Applied Biosystems, USA). For real time PCR, 5 µl of DNA

was added to the 45 µl TaqMan Universal PCR Mastermix Mix (Applied Biosystems, USA) in a final concentrations of 3 pmol/µl of each primer and 2 pmol/µl of the FAM-TAMRA labelled probe. A negative control without added template in the above reaction mixture, was used as a control to detect the presence of contaminating DNA. Amplification and fluorescence detection was conducted in an ABI Prism 7700 sequence detector (Applied Biosystems, USA) with a program of 40 cycles, each cycle consisting of 95°C for 15 seconds and 60°C for one minute as per the manufacturer's instructions.

RESULTS

This study has been conducted to investigate the post flood prevalence of human Leptospirosis in and around Surat. Of the 1,258 suspected cases from Surat, Navsari and Valsad highest incidence 1103 (87.6%) was observed from Surat. In total cases about 801 patients were confirmed with Leptocheck (63.6%), 690 by IgM ELISA (54.8%), 702 by Real Time PCR (55.8%) and 675 MAT (53.6%). The 121 patient's deaths that were reported caused a mortality of 9.61%.

Table 1: Frequency of clinical signs among the suspected cases of leptospirosis from Surat, Navsari and Valsad

Clinical signs	Surat 1103 (87.6%)	Navsari 110 (8.74%)	Valsad 45 (3.57%)	Total *n* = 1258
Fever	1010 (92)	98 (89)	36 (80)	1144 (91)
Mylagia	980 (89)	98 (89)	34 (76)	1112 (88)
Headache	988 (79)	95 (86)	32 (71)	1125 (89)
Jaundice	450 (41)	32 (29)	18 (40)	500 (40)
Nausea/Vomiting	972 (88)	65 (59)	29 (64)	1066 (85)
Meningeal signs	210 (19)	30 (27)	12 (27)	252 (20)
Conjunctival suffusion	740 (67)	28 (25)	8 (18)	776 (62)
Pneumonial/ respiratory	326 (30)	14 (13)	8 (18)	348 (28)
Hemorrhage	678 (61)	28 (25)	16 (36)	722 (57)
Hemoptysis	320 (29)	11 (10)	9 (20)	340 (27)

The most frequent symptom encountered was fever in all the three places; nearly 91% of total cases had fever. Apart from this myalgia, nausea and vomiting, headache and conjunctival suffusion were other common symptoms observed among the patients. Icteric type of illness was associated with 40% of the patients and 57% of patients were reported with severe pulmonary hemorrhages (Table.1).

Table 2: Age and sex wise distribution among the leptospirosis cases during outbreak investigation

Age	Male	Female	Total	%
0-9	17	11	28	2.22
10-19	164	74	238	18.91
20-29	214	144	358	28.45
30-39	189	192	94	30.28
40-49	64	30	94	7.47
50-59	51	41	92	7.31
60-69	44	12	56	4.45
70-79	9	7	16	1.27
80-89	11	3	14	1.11

Age and sex distribution of the patients were analyzed and it revealed most of the patients were in the age group of 10-59 and predominantly males (Table.2). Seven hundred and forty four (59%) were males and five hundred and fourteen were (41%) were females. In this current outbreak situation, the relative risk was estimated to be higher in Surat (33.50), followed by Navsari (19.3) considering the Valsad with minimum number of observed cases as a reference group (Table.3). Seasonal distribution of the cases observed exhibited September (666) as a predominant month followed by August (521), July (50) and October (21) (Table 4). Incidence of leptospirosis observed was higher during heavy rainfall (July-October) in Surat compare to Navsari and Valsad. Crystalline Penicillin 20 lac IU I/V 6 hourly / Rantac I/V 12 hourly was practiced for the treatment of the suspected cases for leptospirosis and it has responded well.

Table 3: Relative risk among the leptospirosis cases of Surat, Valsari and Navsad

Area	No. of cases (%)	Relative risk	Death reported	% Mortality
Surat	1103 (87.6)	33.50	85	6.75
Navsari	110 (8.74)	19.30	29	2.30
Valsad	45 (3.57)	1.0	7	0.55
Total	**1258 (100)**		**121**	**9.61**

Table 4: Month wise distribution of leptospirosis cases during the outbreak investigation

Months	July	August	September	October
Surat	22	453	614	14
Navsari	21	54	31	04
Valsad	07	14	21	03
Total	**50**	**521**	**666**	**21**

The predominant serovars encountered for the outbreak was determined by MAT. Serovars like *autumnalis* (46%), *australis* (38%) and *pyrogenes* (30%) were observed as the predominant circulating serovars with a highest titre of 1:1280 (Table.5). Rapid tests like Leptocheck, Serian IgM ELISA and real time PCR were evaluted in an outbreak situation for leptospirosis (Table 6).

The performances of the rapid test were evaluated based on their sensitivity and specificity of each test in comparison with the gold standard Microscopic Agglutination Test. For Leptocheck WB sensitivity and specificity observed was 91% and 78.4% with a positive and negative predictive value of 83% and 88.3%. For IgM ELISA it was observed as 92.2% sensitivity and 89.4% specificity along with positive and negative predictive value of 90.3% and 91.6%. Among all the three tests the performance of real time PCR was admirable with a sensitivity of 96.5% and specificity of 95.5% and its positive and negative predictive value were determined as 96% and 96%.

Table 5: Distribution of predominant leptospiral serovars among the leptospirosis cases during outbreak investigation

Serovar	Number	%
Autumnalis	357	46
Australis	298	38
Pyrogenes	238	30
Icterohaemorrhagiae	66	8
Cynopteri	158	20
Grippotyphosa	13	1.6
Patoc	13	1.6

Table 6: Evaluation of various diagnostic methods among the Leptospirosis cases during outbreak situation

Tests	Positive cases (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Rapid Leptocheck WB	801 (63.6)	91	78.4	83	88.3
SERION IgM ELISA	690 (54.8)	92.2	89.4	90.3	91.6
Real Time PCR	702 (55.8)	96.5	95.5	96	96

DISCUSSION

The diagnosis of acute undifferentiated febrile illness is difficult in tropical settings where many possible agents can be responsible for infectious disease outbreaks. Such was the case with the outbreak of leptospirosis in Andaman Islands and Nicaragua during the year 1995 [4,5], when thousands of patients developed acute undifferentiated febrile illness and several dozen died of severe pulmonary hemorrhages as the predominant signs and symptoms[8]. Surat is a densely populated area with urbanization combined with industrial developments and prone to garbage and urban wastes that posed a severe impact after this heavy flood. As water receded the entire city was stinking with mud heaps and soon rotten household perishables were also dumped on the streets. The contact between the infectious agent and susceptible individuals can occur distant from the supported foci or the case residence because of rodent and human circulation especially during floods. During the dry periods, high leptospira concentrations in the soil are limited to few meters around the waste accumulation sources. But during the heavy flood conditions it increased the possibilities for the infectious agent to spread and reach a distant area caused by the movement of water. At the same instance, this same flood dilutes both the agent and also its infectivity at a great distance from the sources. This may be evident from our results for the reason by which the Surat city has shown higher relative risk to leptospirosis when compared to other regions like Navsari and Valsad. The scattering of flood water upholds the agent's contact with the population group, so that the individuals with no previous contact with the leptospira and fall under low risk group to leptospirosis may also subjected to infection due to this flood. However, a high prevalence of infection was detected among the individuals living in close proximity and with frequent contact with the agents. Thus, a shift in seropositivity can be predicted in such flood situation over the normal periods. Similar reports were noticed in Reo de Jeneiros, Western region in 1996, where high incidence rates were identified in areas that had precarious sanitation conditions and were vulnerable to floods [18,19]. According to the report, densely populated urban areas displayed an excess of leptospirosis cases around waste accumulation sites. It was observed that in Surat, the incidence was greater during the months of August and September particularly may be because of the deficiency of convenience to the people to reach health care personnel or a hospital under the severe rain fed circumstances and flood havoc. Rather sources of infection may be due to the overflowing of water bodies like ponds, pools, domestic sewage which is often susceptible to urine contamination by the carriers of leptospires like rodents, swine, dogs and cattle.

During this outbreak in and around Surat district of South Gujarat, most of the cases admitted were having high grade fever, headache and generalized body aches, associated with pulmonary hemorrhagic conditions and conjuctival suffusion. Large numbers of cases were observed in Surat city followed the flood with nearly 675 confirmed cases along with 121 deaths. The case fatality rate reported was significant in South Gujarat during the last 13 years of epidemic history. Particularly in patients confirmed with leptospirosis, they were

mostly developed with severe pulmonary haemorrhages in comparison to the previous years. The correlation between clinical forms and the presumptively infecting serovars subsist from previous reports as Australis and Autumnalis usually accompanied by the symptoms like fever, myalgia, and nausea and vomiting, jaundice like signs, conjuctival suffusion and haemorragic conditions[20]. Traditionally, leptospirosis has been considered as a febrile illness. However, they generally remain undiagnosed or are misdiagnosed due to perplexing signs and symptoms, that too under such flood menace marking out the infection becomes extremely complicated unless the disease is suspected in the presence of suggestive epidemiological information. Apart from the environmental risk factors suitable for survival of leptospires, a large population of intermediary hosts like rodents, cattle, dogs and cats which are domesticated by human and susceptible to be in more contact with population during such flood conditions can be an epidemiological niche for frequent transmission of leptospires [21]. Previously studies on human outbreaks have largely relied on serological methods to substantiate clinical cases and to define indirectly the infecting isolate. The standard serological method (MAT) provides a broad idea of serovars responsible for leptospirosis in a given geographic area in spite of the rapid methods like Leptocheck and IgM ELISA. Recently, molecular based methods involving real time PCR has been successfully used to study human outbreaks in Brazil and to characterize isolates recovered from human between 1995 and 2001 in Andaman and Nicobar Islands in India [8]. The requirements of specialized personnel skill for execution, time consuming limitations and maintenance of strains for the preparation of live antigens in laboratory are an everlasting downside of the microscopic agglutination test, although it remains as most widely used reference test. Further the knowledge of the prevalent serovars in a particular geographic area is required as it would be impossible to test with more than 200 pathogenic serovars especially in the situation of such outbreaks under flood havoc conditions. There is an emergency need for a highly sensitive and specific test for early diagnosis of leptospirosis. The sensitivity of these rapid tests usually ranges from 91% to 96.5% and specificity from 78.4% to 95.5%. Identifying leptospirosis as a cause of an outbreak of undifferentiated febrile illness among the population principally after heavy floods in Surat district and the mortality reminds us of the epidemic potential of this disease and its association with particular epidemiologic scenarios. However, the surveillance had emphasized the need for simple, improved and affordable rapid diagnostic tests with high sensitivity and specificity for early diagnosis of leptospirosis that can definitively detect individual patients and thereby tends to reduce mortality rate during the heavy flood endemic periods. The deployment of rapid molecular approaches like real time PCR can be very well considered for such endemic circumstances to efficiently overcome the difficulties tied up with basic serological methods.

REFERENCES

1. World Health Organization. Leptospirosis worldwide. Wkly Epidemiol Rec. 1999; 74: 237-242.
2. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001; 14: 296-326.
3. Meslin FX. Global aspects of emerging and potential zoonosis: a WHO perspective. Emerg Infet Dis. 1997; 3: 223-228.
4. Trevejo RT, Rigau PJG, Ashford DA, McClure EM, Jarquin GC, Amador JJ et al. Epidemic leptospirosis associated with pulmonary hemorrhage – Nicaragua, 1995. J Infect Dis. 1998; 178: 1457-1463.
5. Centre for Disease Control and Prevention. Outbreak of acute febrile illness and pulmonary hemorrhage-Nicaragua. MMWR Morb Mortal Wkly Rep. 1995; 44: 839-843.
6. Bharadwaj R, Bel AM, Joshi SA. An urban outbreak of leptospirosis in Mumbai, India. Jpn J Infect Dis. 2000; 55:194-6
7. Anuchai N, Kannika N, Galayanee D. Surviellance of leptospirosis after flooding at Loei Province, Thailand. Southeast Asian J Trop Med Public Health. 2005; 36:203-205.
8. Vijayachari P, Sehgal SC, Marga G, Goris A. Terpstra WJ. Hartskeeri RA. Leptospira interrogans serovars valbuzzi: a cause of severe pulmonary haemorrhages in the Andaman Islands. J Med Microbiol. 2000; 5: 1-6.
9. Faine SB, Adler B, Bolin C, Perolat P. Leptospira and Leptospirosis. Melbourne MediSci. 1999.
10. Centre for Disease Control and Prevention. Outbreak of leptospirosis among white-water rafters-Costa Rica, 1996. MMWR Morb Mortal Wkly Rep. 1997; 46: 577-579.
11. Ko AI, Reis MG, Ribeiro DCM, Johnson WD Jr, Riley LW. Urban epidemic of severe leptospirosis in Brazil. Lancet. 1999; 354: 820-825.
12. Caldas EM, Costa E, Sampaio MB. Leptospirosis in Salvador (Brazil). Clinical and laboratory aspects. Rev Inst Med Trop Sau Paulo. 1978; 20: 164-176.
13. Sakata EE, Yasuda PH, Romero EC, Silva MV, Lomar AV. The serovars of Leptospira interrogans isolated from cases of human leptospirosis in San Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 1992; 34: 217-221.
14. Marotto PC, Nascimento CM, Eluf-Neto J, Marotto MS, Andrade L, Sztanjnbok J, et al. Acute lung injury in leptospirosis: clinical and laboratory features, outcome and factors associated with mortality. Clin Infect Dis. 1999; 29: 1561-1563.
15. Goncalves AJ, deCarvalho JE, GuedeseSilva JB, Rozembaum R, Vieira AR. Hemoptysis and the adult respiratory distress syndrome as the causes of death in leptospirosis. Changes in the clinical and anatomicopathological patterns. Rev Soc Bras Med Trop. 1992; 25: 261-270.
16. Angela PB, Eide DC, Emilson DS, Marcos VS, Rui VA. Macroscopic Agglutination Test for Rapid Diagnosis of Human Leptospirosis. J Clin. Microbiol. 1998; 36: 3138-3142.
17. Cole JR, Sulzer CR Jr, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol. 1973; 25: 976-980.
18. Barcellos C, Sabroza PC. The place behind the case: leptospirosis, risks and associated environmental conditions in a flood-related outbreak in Rio de Janeiro. Cad Saude publica. 2001; 17: 59-67.
19. Kupek E, deSousa SFMC, deSouza PJM. The relationship between rainfall and human leptospirosis in Florianopolis, Brazil, 1991- 1996. Braz J Infect Dis. 2000; 4: 131-134.
20. Ratnam S, Subramanian S, Madanagopalan N, Sundaraj T, Jayanthi V. Isolation of leptospires and demonstration of antibodies in human leptospirosis in Madras, India. Trans Roy Soc Trop Med Hyg. 1983; 77:455-458.
21. Natarajaseenivasan K, Boopalan M, Selvanayaki K, Raja SS, Ratnam S. Leptospirosis among rice mill workers of Salem, South India. Jpn J Infect Dis. 2002; 55: 170-173.

ISSN: 0976 3325

Original Article

SEROPREVALENCE OF LEPTOSPIROSIS IN SOUTH GUJARAT REGION BY EVALUATING THE TWO RAPID COMMERCIAL DIAGNOSTIC KITS AGAINST THE MAT TEST FOR DETECTION OF ANTIBODIES TO LEPTOSPIRA INTERROGANS

Tanvi Panwala[1] Summaiya Mulla[2] Parul Patel[3]

[1]Assistant Professor [2]Professor & Head, Department of Microbiology, Government Medical College, Surat [3]Tutor, Department of Microbiology, GMERS, Sola, Ahmedabad

Correspondence:
Dr. Tanvi Panwala
E-7, Vigneshawar Estate,
Nanpura, Timaliyavad, Surat-395001
E-mail : drtanvi2006@yahoo.co.in, Mobile no. : 98258-31144

ABSTRACT

The study was conducted to evaluate the two rapid tests for the serologic diagnosis of leptospirosis namely Microplate Immunoglobulin M(IgM)-Enzyme Linked Immunosorbent Assay(ELISA) and IgM Rapid Leptocheck WB and the performance of each assay compared with that of the current standard, the microscopic agglutination test (MAT). The panels of 188 sera from 130 cases of leptospirosis from three different geographical locations were tested as well as 310 sera from healthy individual or individual with other infectious disease other than leptospirosis. Acute phase sera from cases (n=130) were collected <14 days after the onset of symptoms and convalescent phase sera (n=58) were collected ≥14 days after the onset of symptoms. By traditional method (two-by-two) contingency table, the sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid Leptocheck WB were 98.36%, 86.95%, 86.95%, 98.36%, 92.37% and 0.81 in acute phase of disease. Corresponding values for IgM ELISA were 96.82%, 88.05%, 88.40%, 96.72%, 91.53% and 0.88 respectively. The sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid Leptocheck WB were 87.87%, 88%, 90.82%, 84.61%,86.20% and 0.85 in convalescent phase of the disease. Corresponding values for IgM ELISA were 91.42%, 95.65%, 96.96%, 88%, 93.10% and 0.81 respectively. These values for the 2 tests were comparable, indicating that there was no difference in their efficacies. The second-generation assay included in study (Leptocheck and ELISA) showed significantly higher sensitivity with early acute phase sera than the reference or first generation method (MAT) while retaining high specificity and should greatly improve the rapid detection of leptospirosis in the field.

KEY WORDS: Leptospirosis, MAT test, IgM ELISA test, IgM Rapid Leptocheck test.

INTRODUCTION

Leptospirosis is a zoonosis caused by spirochetes of the genus *Leptospira*, which has a worldwide distribution[1]. Humans become infected through contact with contaminated animal urine, tissues, or water[2] The clinical presentation is difficult to distinguish from dengue, malaria, influenza, and many other diseases characterized by fever, headache, and myalgia[3]. Although the patient's exposure history may assist in narrowing the differential diagnosis, a rapid and simple test with high sensitivity and specificity would be useful for early diagnosis and treatment and for public health surveillance[4]. Definitive laboratory diagnosis of leptospirosis requires detection of the organism in a clinical specimen or a fourfold or greater rise in microscopic agglutination test (MAT) titer in the setting of an appropriate clinical syndrome.

The most frequently used diagnostic approach for leptospirosis has been that of serology. The

MAT is the serological test used in reference laboratories, because of its high degree of sensitivity and specificity[5]. However, the MAT is a complex test that requires a large panel of live-cell suspensions to provide adequate coverage of the antigenic diversity represented in a given testing area. Moreover, antibody levels detectable by MAT usually do not appear before day 6 or 7 after development of symptoms; they usually peak by the fourth week, but detectable titers may persist for years[6, 7, 8]. Hence, interpretation of the results is difficult without paired specimens collected at the appropriate times; therefore, results are usually not available quickly enough to be useful for patient management.

Several alternatives to the MAT have been developed; those available commercially include an Immunoglobulin M (IgM) Enzyme-Linked Immunosorbent Assay (ELISA)[9], an IgM dipstick assay (LDS)[10], an IgM dot-ELISA dipstick test (DST)[11], and the indirect heamagglutination assay (IHA)[12]. Reported evaluations suggest that some of these assays are highly sensitive and specific[12, 13, 14, 15, 16, 17, 18], but they have not been systematically compared to each other and to the MAT. This study was designed to determine the performance of these serologic assays in detecting *Leptospira*-specific antibodies and to compare results obtained with each system to those obtained with the MAT. This information should assist diagnostic laboratories, especially those without the capacity to maintain the MAT, to select a suitable assay for screening serum samples from suspected cases of leptospirosis.

MATERIAL AND METHODS

Case sera: The study was conducted at new civil hospital, Surat, India, a tertiary health centre in South Gujarat during the period May 2007 to July 2008. All suspected cases of Leptospirosis attending the outpatient department of these hospitals during the study period were included. A total of 188 sera from 130 cases were included in the study, the panel of case sera (188 specimens) consisted of 130 acute phase sera (obtained <14 days after the onset of illness) and 58 convalescent phase sera (obtained 14 to 28 days after the onset of illness). Paired sera were available for 58 cases. Samples were from different geographic location namely, 76 cases were from Surat district, 18 cases were from Valsad district and 36 cases were from Navasari district. **Control sera:** A total of 310 control specimen were collected which includes 50 healthy donors, 100 were from individuals known to have disease other than leptospirosis and 160 healthy control from different geographic locations. Information helpful in the interpretation of results such as agent or disease specific finding and place of residence was obtained.

Criteria for clinical suspicion of leptospirosis: Acute febrile illness with headache, myalgia and prostration associated with any of the following:

- Conjuctival suffusion
- Meningeal irritation
- Anuria or oliguria and/or proteinuria
- Jaundice
- Hemorrhages (from the intestines; lung bleeding is notorious in some areas)
- Cardiac arrhythmia or failure
- Skin rash and a history of exposure to infected animals or an environment contaminated with animal urine.
- Other common symptoms include nausea, vomiting, abdominal pain, diarrhea & arthralgia.

MAT test: The MAT test was performed using standard procedure[19]. Live leptospira (representing 11 serovars belonging to 11 serogroup) cultured in EMJH (Ellinghausen-McCullough- Johnson-Harris) media to detect agglutination antibodies from patient sera. Live leptospira cell suspension were added to serially diluted serum specimens in 96 well flat bottomed microtiter plates and incubated at 37^0C for 2 hours. Agglutination was examined by dark field microscopy at a magnification of 100X. The reported titer was calculated as the reciprocal of the highest dilution that agglutinated at least 50% of the cells for each serovar.A MAT test is considered borderline at titre of $\geq$80 and positive at titre of $\geq$200 for single samples. Serogroup included in the antigen panel are as follows:
Australis (Australis), Autumnalis (Bangkinang), Ballum (Ballum), Sejroe (Hardjo), Grippotyphosa (Grippotyphosa), Canicola (Canicola), Hebdomadis (Hebdomadis), Pomona (Pomona), Semeranga (Patoc1), Pyrogen (Pyrogen), Icterohaemorrhagiae (Icterohaemorrhagiae).

IgM ELISA test: The ELISA was carried out as per the manufacturer's instruction. ELISA kit was obtained from Serion verion ELISA (classic leptospira IgM). Serum antibodies of the IgM

class, when present, combine with leptospira antigen attached to the polystyrene surface of the microwell test strips. Residual serum is removed by washing and peroxidase conjugated antihuman IgG, IgA, IgM is added. The microwells are washed and substrate system, para-nitrophenyl-phosphate is added. The substrate is hydrolysed by enzyme, and chromogen changes to yellow coloured. Case and control sera (10µL) were diluted 1:100 and tested according to the manufacturer's instruction. The result is read with a dual wavelength spectrophotometer at 405nm and a background of 620nm. The colour intensity is directly related to the concentration of Leptospira IgM antibodies in the test sample. Each set of tests is run with a positive control, negative control and cut-off calibrator in duplicate. The test is valid when the absorbance reading of the above meets the specification of the Serion ELISA instruction. The results were interpreted according to the manufacturer's recommendation. Specimens having an absorbent ratio greater than that of cutoff calibrator were defined as positive.

Calculation for Serion ELISA classic leptospira IgM:

- Serion units of <15 gives a negative result interpreted as no evidence of recent infection.
- A Serion unit of 15-20 is a low positive or borderline result and may suggest a recent infection.
- Serion units of >20 is a positive result suggestive of a recent or current infection.

Samples giving borderline results should be tested in parallel with a further sample taken from the patient 1-2 weeks later.

Rapid Leptocheck Test: Case and control sera (10µL) were used and tested according to the manufacturer's instruction. It utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flow through the membrane assembly of the test device, the anti-human IgM colloidal gold conjugate forms a complex with IgM antibodies in the sample. This complex moves further on the membrane to the test window 'T' where it is immobilized by the broadly reactive leptospira genus specific antigen coated on the membrane, leading to the formation of a red to deep purple coloured band at the test region. 'T' which confirms a positive test result. Absence of this coloured band in test region 'T' indicates a negative test result. The unreacted conjugate and the unbound complex if any move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies, coated on the control window "C" of the membrane assembly, forming a red to deep purple coloured band. The control band shows to validate the test result.

Criteria for laboratory confirmation: The suspected patients fulfilling any of the following criteria were considered as a case of leptospirosis:(1) isolation of leptospira from clinical specimen (2) Seroconversion in IgM ELISA and MAT test from seronegative to a titre of at least 100, (3) Fourfold or greater increase in MAT or ELISA titre between acute and convalescent phase serum specimens obtained 2 weeks apart and studied at the same laboratory (4) a titre of >100 in IgM ELISA or >200 in MAT if only a single sample was available.

DATA ANALYSIS

Sensitivity, specificity, positive predictive values(PPV), negative predictive values(NPV), Kappa value were calculated based on MAT cutoff of $\geq$80 dilution , using standard equations:

- % sensitivity =true positive / (true positive + false negative) × 100.
- % specificity = true negative/ (false positive + true negative) × 100.
- PPV (Positive predictive value) = true positive/all positive test.
- NPV (Negative predictive value) = true negative/ all negative test.
- Efficiency of test= (true positive +true negative)/total samples

RESULTS

The sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid Leptocheck WB were 98.36%, 86.95%, 86.95%, 98.36%,92.37% and 0.88 in acute phase of disease. Corresponding values for IgM ELISA were 96.82%, 88.05%, 88.40%, 96.72%, 91.53% and 0.88 respectively. These values for the 2 tests were comparable, indicating that there was no difference in their efficacies.

The sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid

Leptocheck WB were 87.87%, 88%, 90.82%, 84.61%,86.20% and 0.85 in convalescent phase of the disease. Corresponding values for IgM ELISA were 91.42%, 95.65%, 96.96%, 88%, 93.10% and 0.81 respectively. So, the changes in the values of these tests, depending on the stage of the disease are shown in table-1 and chart- 1& 2 below.

Table 1: Comparison of two rapid tests in acute and convalescent phase

Tests	Phases	Sensitivity	Specificity	PPV	NPV	Efficiency
Leptocheck WB	**Acute Phase (< 14 days)**	98.36%	86.95%	86.95%	98.36%	92.37%
	Convalescent phase (14-28 days)	87.87%	88.00%	90.62%	84.61%	6.20%
IgM ELISA	**Acute Phase (< 14 days)**	96.82%	88.05%	88.40%	96.72%	91.53%
	Convalescent phase (14-28 days)	91.42%	95.62%	96.96%	88.00%	93.10%

The sensitivity of the MAT for diagnosis of leptospirosis was also tested which showed sensitivity of 44.61% during 1st week and 60.38% during second to fourth week. These values were lower than the corresponding values for the Leptocheck WB and IgM ELISA.

DISCUSSION

Leptospirosis is an acute febrile disease, widely recognized as being emergent or re-emergent in tropical and subtropical regions, the disease is endemic and exposure to infection is widespread. In temperate climates, the disease is primarily one of occupational, recreational expose. Leptospirosis is frequently under-diagnosed, because of the non-specific symptoms early in the disease and the difficulty of performing the culture.

In leptospirosis, antibodies begin to appear within a few days of onset of symptoms and in a significant proportion of patients the antibodies persist in detectable quantities for several months (Silva et al, 1995). As has been described, genus specific antibodies appear earlier than the serovar specific microscopic agglutinating antibodies. At this earlier stage of the disease, genus-specific tests, especially IgM immunoassays, are expected to be positive though more serovar specific tests such as MAT may not be able to detect the presence of antibodies owing to nil or low immune response (Christie, 1980). From the clinical point of view, the ability to detect the infection early in the course of the disease is of extreme importance for initiating appropriate treatment to avoid serious complications. In this context, the genus specific IgM immunoassays would be of great use for detecting leptospirosis at an early stage of the disease.

One of the drawbacks of IgM immunoassays and Rapid Leptocheck WB is their inability to give any information about the infecting serovars. But such information is mainly of epidemiological importance, as differentiation between the infecting serovars does not affect the clinical course of management. The usefulness of these rapid genus-specific immunoassays is at the peripheral level, where the only information required is whether or not a patient has a leptospiral infection.

The sensitivities of both rapid Leptocheck WB and IgM ELISA are at acceptable levels even during the first week of illness when the IgM antibodies start to appear. This indicates that the assays are highly responsive to even low levels of IgM antibodies. As the tests have high PPV during all stages of the disease, these tests are useful for screening. Since these tests detect IgM antibodies, which persist for a shorter period than IgG antibodies, their NPV begin to decline after 1 month of infection. Because of this, these tests will have only limited usefulness in epidemiological studies on prevalence of infection among a population.

As MAT detects both IgM and IgG antibodies, it is difficult to differentiate between current clinical infection and past exposure to leptospira using a single MAT. In this regard there is a need to define criteria for a positive MAT when MAT is used alone for serodiagnosis of leptospirosis. Based on our criteria, MAT on a single sample had shown only 44.61% sensitivity during the acute phase (0 to 14 days) of illness. This comparatively ≥1:80 cut-off value was used

because the study was conducted in an endemic zone with high seroprevalence among the healthy population. The sensitivity of MAT rose to 60.38% during the convalescent phase (14 to 28 days) of disease. Some of the patients who had negative MAT results during the first weeks of disease and they became positive by seroconversion and showed rising titres when another sample obtained 14 days after the onset of illness was examined.

Therefore, this test is a useful tool for epidemiological purpose.

- We observed that more patients were male in our study. Almost are working class male farm workers.
- We observed that there were 71 (seventy one) i.e. more number of cases in the age group of 20-39 years. This reflects as they are active earning adult age groups and from history majority of these had occupational history as farmer.

Among the 100 serum samples from patients with disease other than leptospirosis (malaria, dengue, hepatitis, typhoid, HIV). There were no false positive reactions observed with Leptocheck WB or IgM ELISA. It may be due to we used limited numbers diseased groups. We did not observe any significant difference in the cross-reactivity rate in different disease by ELISA & Leptocheck WB. None of the sera from the above groups of patients had given significant titres by MAT. However, low titres by MAT (1:20-1:40) were obtained for some of the patients, which reflects that it may be IgG antibody.

Table 2: Results of our study in comparison with other studies

Test	Results	WYsekhar EH Soo[4, 8]	SC Sehgal, PV Vijaychari[4, 2]	Present study
Rapid test Leptocheck or Dipstick	Sensitivity	83.3%	78.7%	93.81%
	Specificity	93.8%	88.3%	86.81%
	PPV	95.29%	91.0%	88.34%
	NPV	79%	73.4%	92.94%
IgM ELISA	Sensitivity	54.2%	78.5%	93.81%
	Specificity	96.9%	87.6%	90.10%
	PPV	96.3%	90.5%	91.00%
	NPV	58.5%	73%	93.81%

Our study was compared with other studies (table- 2), our study sensitivity for rapid test is 94.68 % which is comparable to the other two studies (WY Sekhar, EH, Soo[20], P. Vijayachari et al[21]). It is slightly higher than the other two studies which may be due to the difference in test as they have used Dipstick as a rapid method which is based on immunochromatography principal, and in our study we have used Leptocheck WB (lateral flow method).

The specificity of P. Vijayachari et al[21] & W.Y. Sekhar EH Soo[20] ranges from 88% to 94%. In our study, it was 87.23% which correlates well with their studies.

In case of IgM ELISA, the sensitivity of WY Sekhar study was very low, which may be due to difference in kit mode. They have used PanBio for their study, where as we have used Serion Virion IgM ELISA which was evaluated according to Indian geographical areas.

The sensitivity of P. Vijayachari study was also slightly lower than our study but it is comparable. The specificity of two studies correlates well with our study.

The agreement between Rapid test with MAT and IgM ELISA with MAT test were 80% and 84% respectively which are comparable to SC Sehgal, P. Vijayachari et al study.

Additionally one of the major limitations for any evaluation of assays for serologic diagnosis of leptospirosis is the paucity of cases confirmed by culture. As a result, findings from new serologic assays are comparable with those from cases that are primarily defined by another serologic assay. Consequently, there are very few reports of sensitivity and specificity of the MAT, because it is the gold standard against which other assays are usually compared.

CONCLUSION

This study was conducted at New Civil Hospital, Surat during the period May 2007 to July 2008. There were 130 clinically suspected cases from different regions of South Gujarat. Majority of patients were young adults. There was male preponderance, and majorities were farm workers.

The Rapid Leptocheck WB test is easy to perform and it requires only a single dilution and does not require any special equipment. The kit reagents have a long shelf-life even at room temperature. The test has good sensitivity (98.36%) and specificity (86.95%) in acute phase and sensitivity of 87.87% and specificity of 88% in convalescent phase considering MAT as Gold Standard. So, it is now the test of choice for the diagnosis of current leptospirosis, and for routine use at the peripheral level in developing countries. IgM ELISA is also very good test for early detection of leptospiral infection which has good sensitivity (96.82%) and specificity (88.05%) in acute phase and sensitivity of 91.42% and specificity of 95.62% in convalescent phase considering MAT as Gold standard. The limitation of this test includes its ability to give information about the infecting serovar because of these both are genus-specific nature. Therefore MAT test is a useful tool for epidemiological purpose.

The microscopic agglutination test (MAT) (WOLFF, 1954) is still the 'corner-stone' of leptospirosis diagnosis. However, the test has many disadvantages. Considerable laboratory infrastructure and skilled manpower are required for performing MAT. Many strains of leptospires have to be maintained in the laboratory for use as antigens in the test. Standardisation of the test can detect both IgM and IgG antibodies, but it may fail to demonstrate low levels of IgM antibodies during the early stage of the disease. The value of MAT lies in its ability to recognize the infecting serogroup, especially in repeat sample collected 10-14 days after the first specimen. Therefore, this test is a useful tool for epidemiological purposes.

So, the second-generation assay included in our study (Leptocheck and ELISA) showed significantly higher sensitivity with early acute phase sera than the reference or first generation method (MAT) while retaining high specificity and should greatly improve the rapid detection of leptospirosis in the field.

REFERENCE

1. World Health Organization. 1999. Leptospirosis worldwide, 1999. Wkly. Epidemiol. Rec. 74:237-242.
2. Levett, P. N. 2001. Leptospirosis. Clin. Microbiol. Rev. 14:296-326.
3. Tappero, J. W., D. A. Ashford, and B. A. Perkins. 2000. Leptospira species (leptospirosis), p. 2495-2501. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases, 5th ed. Churchill Livingstone, Philadelphia, Page.34
4. Perkins, B. A. 1998. Epidemic leptospirosis associated with pulmonary hemorrhage in Nicaragua, other recent outbreaks, and diagnostic testing: issues and opportunities, p. 159-167. In W. M. Scheld, W. A. Craig, and J. M. Hughes (ed.), Emerging infections 2. American Society for Microbiology, Washington, D.C.25
5. Cole, J. R., C. R. Sulzer, and A. R. Pursell. 1973. Improved microtechnique for the leptospiral microscopic agglutination test. Appl. Microbiol. 25:976-980.
6. Adler, B., S. Faine. 1978. The antibodies involved in the human immune response to leptospiral infection. J. Med. Microbiol. 11:387-400.
7. Cumberland, P. C., C. O. R. Everard, J. G. Wheeler, and P. N. Levett. 2001. Persistence of anti-leptospiral IgM, IgG and agglutinating antibodies in patients presenting with acute febrile illness in Barbados 1979-1989. Eur. J. Epidemiol. 17:601-608.
8. Terpstra, W. J., G. S. Ligthart, and G. J. Schoone. 1985. ELISA for the detection of specific IgM and IgG in human leptospirosis. J. Gen. Microbiol. 131:377-385.
9. Winslow, W. E., D. J. Merry, M. L. Pirc, and P. L. Devine. 1997. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J. Clin. Microbiol. 35:1938-1942.
10. Gussenhoven, G. C., M. A. W. G. van der Hoorn, M. G. A. Goris, W. J. Terpstra, R. A. Hartskeerl, B. W. Mol, C. W. Van Ingen, and H. L. Smits. 1997. LEPTO dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human sera. J. Clin. Microbiol. 35:92-97.
11. Levett, P. N., S. L. Branch, C. U. Whittington, C. N. Edwards, and H. Paxton. 2001. Two methods for rapid serological diagnosis of acute leptospirosis. Clin. Diagn. Lab. Immunol. 8:349-351.
12. Levett, P. N., and C. U. Whittington. 1998. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis. J. Clin. Microbiol. 36:11-14.
13. Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. Clin. Infect. Dis. 34:1593-1599.
14. Brandão, A. P., E. D. Camargo, E. D. da Silva, M. V. Silva, and R. V. Abrão. 1998. Macroscopic agglutination test for rapid diagnosis of human leptospirosis. J. Clin. Microbiol. 36:3138-3142.
15. Cinco, M., D. Balanzin, and E. Banfi. 1992. Evaluation of an immunoenzymatic test (ELISA) for the diagnosis of leptospirosis in Italy. Eur. J. Epidemiol. 8:677-682.
16. Ribeiro, M. A., C. C. Souza, and S. H. P. Almeida. 1995. Dot-ELISA for human leptospirosis employing immunodominant antigen. J. Trop. Med. Hyg. 98:452-456.
17. Silva, M V, PM Nakamura, E D Camargo, L Batista, A J Vaz, E C Romero, A P Brandão. Immunodiagnosis of human leptospirosis by dot-ELISA for the detection of IgM, IgG, and IgA antibodies. Am. J. Trop Med Hyg 1997;56:650-655.
18. Yersin, C., P. Bovet, H. L. Smits, and P. Perolat. 1999. Field evaluation of a one-step dipstick assay for the

diagnosis of human leptospirosis in the Seychelles. Trop. Med. Int. Health 4:38-45.
19. Vijayachari P, Suganan 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.
20. WY Sekhar, E H Soo, V Gopalkrishnan, S Devi. Leptospirosis in Kuala Lumpur and the Comparitive Evaluation on of two Rapid Commercial Diagnostic Kits Against the MAT test for the Detection of antibodies to leptospira Interrogans. Singapore Med J 2000; 41(8):373
21. Sehgal SC, Vijaychari P, Sharma S, Sugunan AP. Leptodipstick –A rapid and simple method for serodiagnosis of leptospirosis in acute stage. Trans Soc Trop Hyg 1999; 93:1-4.

DOI: 10.7860/JCDR/2015/11188.5587

Original Article

Microbiology Section

To Evaluate the Different Rapid Screening Tests for Diagnosis of Leptospirosis

TANVI PANWALA[1], SANGEETA RAJDEV[2], SUMMAIYA MULLA[3]

ABSTRACT

Introduction: Leptospirosis is an acute febrile disease, in tropical and sub-tropical regions of world. It has been under-reported in India, due to presence of non-specific symptoms and unavailability of appropriate laboratory diagnostic facilities in most part of the country. The diagnosis of leptospirosis is usually based on demonstration of antibodies by different serological tests.

Aim: The present study aims to evaluate and compare commercially available rapid test.

Design and Settings: Case control study.

Materials and Methods: Three screening tests (Leptocheck WB, Latex agglutination test and SD leptospira) were compared by using 100 serum samples randomly obtained from clinical cases of Leptospirosis admitted in new civil hospital, Surat, Gujarat. All the patients with acute Leptospirosis were included in this 4-months pilot study from July 2011 to October 2011. All the results were compared with IgM ELISA and MAT for confirmation of diagnosis.

Results: Leptocheck WB, Latex agglutination test and SD leptospira had sensitivities of 84.8%, 84.8% and 72.7% & specificities of 37.3%, 71.2% and 71.2% respectively as compared to MAT. Leptocheck WB, Latex agglutination test and SD leptospira had sensitivities of 90.7%, 89.7% and 53.7% & specificities of 93.4%, 90.9% and 60% respectively as compared to IgM ELISA.

Conclusion: Latex agglutination test kit and Leptocheck WB were found to be highly sensitive and specific. Neither of these tests require specialized equipment, and could be performed in peripheral laboratories with relatively little expertise.

Keywords: IgM ELISA, Leptospirosis, Leptorapide, Leptocheck, MAT, SD leptospira IgM/IgG

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by spirochetes of the genus Leptospira [1,2]. The disease is endemic in some tropical and subtropical region and exposure to infection is widespread [3]. Leptospirosis is characterised by wide clinical variability, ranging from a mild flu-like illness to an acute life threatening condition, but only patients with the symptomatic forms of the disease are hospitalised [4]. Leptospirosis is a common cause of acute febrile illness in tropical climate and must be differentiated from other infection like typhoid, malaria, dengue, scrub typhus, viral hepatitis etc [5].

Early diagnosis of Leptospirosis is important since mortality rate is high in patient with severe Leptospirosis [5]. Diagnosis of Leptospirosis is often made by serological tests. The MAT is the serological test used in reference laboratories because of its high degree of sensitivity and specificity [2]. However, MAT is a complex test that requires significant expertise and large panel of live-cell suspensions, as well as, antibody levels detectable by MAT usually appear after day 6 or 7 of symptoms. Hence, interpretation of the results is difficult and results are usually not available quickly enough for patient management [2,5].

The early diagnosis of Leptospirosis is now possible by using different serological methods which are available commercially in the market such as an IgM ELISA, an IgM dipstick assay (LDS), latex agglutination test, lepto lateral flow test and the indirect hemagglutination assay (IHA) [2,6]. Therefore, rapid and easy to perform tests have emerged in recent years for the diagnosis of Leptospirosis. Majority of these rapid tests are immunochromatographic or particle agglutination tests. The introduction of such tests in the market needs their evaluation by comparing their results with the gold standard MAT or other tests like IgM ELISA [7]. Aim of the present study was to evaluate the usefulness of the diagnostic test kits (leptocheck WB, Latex agglutination test and SD leptospira) for the diagnosis of Leptospirosis by comparing their results with the 'gold standard' test, MAT and IgM ELISA.

MATERIALS AND METHODS

Patients and sera: Serum specimens from 100 patients enrolled randomly in the study, conducted from July 2011 to October 2011. All the patients with acute Leptospirosis admitted in New Civil Hospital, Surat, Gujarat were included in this 4-months pilot study. Clinical suspicion of acute Leptospirosis was defined as fever and/or myalgia, tender liver, jaundice, acute renal failure, bleeding tendency, meningism and radiological lung infiltrates which accounted in the first week of fever. The study was approved by ethical committee of the institute. All the serum samples were tested for three commercially available rapid kits; Leptocheck WB, Latex agglutination test and SD leptospira. All the results were compared with IgM ELISA and MAT for confirmation of diagnosis.

MAT test: The MAT test was performed using standard procedure [8]. Serogroups included in the antigen panel were: *L.Australis (Australis), L.Autumnalis (Bangkinang), L.Ballum (Ballum), L.Sejroe (Hardjo), L.Grippotyphosa (Grippotyphosa), L.Canicola (Canicola), L.Hebdomadis (Hebdomadis), L.Pomona (Pomona), L.Semeranga (Patoc1), L.Pyrogen (Pyrogen), L.Icterohaemorrhagiae (Icterohaemorrhagiae).* All the strain were obtained from National Leptospirosis Reference Centre, RMRC, WHO collaborating centre, ICMR, Portblair. These serovars were maintained in semisolid 0.1% EMJH (Ellinghausen-McCullough-Johnson-Harris) agar by using Leptospira medium base supplemented with 10% enrichment (Difco,USA) at 28-30ºC. Doubling dilution of serum in 96 well flat bottomed microtitre plates from 1 in 25 to 1 in 1600 was prepared

by using phosphate buffer saline suspension as diluents. 50 µl of the specific serovar (Mc Farland 1.0) added to all wells. One of the wells with antigen only, without addition of antibody served as the antigen control. The final dilution after adding the antigen was 1 in 50 to 1 in 3200.The plate was covered with aluminium foil and incubated at 37°C for 2 h in wet chamber or humid chamber to avoid dehydration. After 2 h of incubation, slide was examined by dark field microscopy at a magnification of 40X. The highest serum dilution showing approximately 50% agglutinated leptospires or reduction in the number of leptospiral cells as compared to the antigen control was taken as end point titer. MAT test is considered positive at titre of ≥100 for single serum samples [8,9].

Pan bio LeptospiraIgM ELISA test: Whole procedure was performed according to manufacturer's instruction. Test sera and controls were diluted in 1:100 in serum diluents and 100 µl added into Leptospira (serovar patoc) antigen coated microwell. Then plate was incubated for 30 min at 37°C. After washing the plate with phosphate- buffered saline solution, 100 µl of HRP-conjugated anti-human IgM added and incubated for further 30 min at 37°C. Again washing the plate with buffered solution, 100 µl of the TMB (tetramethylbenzidine) substrate was added and incubated for 10 min at room temperature. Then reaction was stopped with 100 µl of 1M phosphoric acid. The absorbance value of each well was read at 450 nm wave length and reading was interpreted in terms of Pan-Bio units which in turn were calculated by the absorbance of positive control serum, negative control serum and cut-off of calibrators provided by the manufacturer. Pan Bio unit ≥11 was considered positive [2].

Rapid Leptocheck Test (Lot no.: 51080): Case and control sera (10µL) were used and tested according to the manufacturer's instruction. It utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flow through the membrane of the test device, the anti-human IgM colloidal gold conjugate forms a complex with IgM antibodies in the sample. This complex moves further on the membrane to the test window 'T' where it is immobilized by the broadly reactive leptospira genus specific antigen coated on the membrane, leading to the formation of a red to deep purple coloured band at the test region. 'T' which confirms a positive test result. If there is no band at the test region, it indicates negative result. At the 'C' window, the anti-rabbit antibodies is coated and the unreacted conjugate and the unbound complex if any move further on the membrane and are subsequently immobilized here and forming a red to deep purple coloured band. If there is no control band, it suggests the test is invalid [10].

Leptorapide (Latex agglutination test- Lot no. 230511-01): Whole test was performed according to the manufacturer's instruction. 5 µl of Leptorapide reagent was added by dispensing pipette on the agglutination card. Then add 5 µl of test sera with new dispensing pipette to the 5 µl Leptorapide reagent and mix. Agglutination card was rotated gently for 2-3 min and a result was interpreted by using score card. A positive/negative result will appear within 3 min of mixing. Score extent of agglutination according to the scale [11].

SD LeptospiraIgM/IgG (Lot no. 99004): Whole test was performed according to the manufacturer's instruction. Allow all kit components and specimen to room temperature prior to testing. Test device was removed from foil pouch and placed it on a flat, dry surface. 5µl of serum or plasma specimen was added into the square sample well marked as "S". Four drops of assay diluent was added to the assay diluent well which is round shaped. Test results were interpreted within 20 min [12].

RESULTS

Total 100 samples were evaluated. 80 were IgM ELISA positive and 20 were IgM ELISA negative. 28 were MAT positive and 72 were MAT negative. All the samples were tested for three commercially available rapid kits Leptocheck WB, Latex agglutination test leptorapide and SD leptospira. Leptocheck WB has given 52 positives and 48 negative results; Latex agglutination test has given 72 positives and 28 negatives while SD leptospira rapid kit has given 51 positives and 49 negatives. Results of tests were compared considering ELISA and MAT as gold standard. Sensitivity, specificity, PPV, NPV and accuracy of three rapid tests were determined in comparison to IgM ELISA and MAT. Leptocheck WB, Latex agglutination test and SD leptospira had sensitivities of 84.8%, 84.8% and 72.7% & specificities of 37.3%, 70.1% and 70.1% respectively as compared to MAT. Leptocheck WB, Latex agglutination test and SD leptospira had sensitivities of 90.7%, 89.7% and 53.7% & specificities of 93.4%, 90.9% and 60% respectively as compared to IgM ELISA. Comparisons of results are shown in [Table/Fig-1-4] for MAT & IgM ELISA respectively.

DISCUSSION

As the disease Leptospirosis shows protean clinical manifestations, laboratory confirmation is a must. Isolation of leptospiras from clinical samples is time consuming; serology remains the mainstay of diagnosis [7]. MAT is not rapid test and it is used mainly in the reference laboratory only. In addition, its role in early diagnosis is rarely available. Various kits for rapid detection of Leptospirosis available commercially are simple, convenient, rapid and do not need complicated laboratory equipment. Moreover they do not require skilled hands and thus prove to be a suitable option for diagnosis in the peripheral regions. Though MAT and ELISA tests are widely used for confirmation of Leptospirosis, these commercially available rapid tests are also found to be effective. Thus their sensitivity and specificity needs to be evaluated by comparing them with ELISA and MAT results keeping them as references (gold standard) [4].

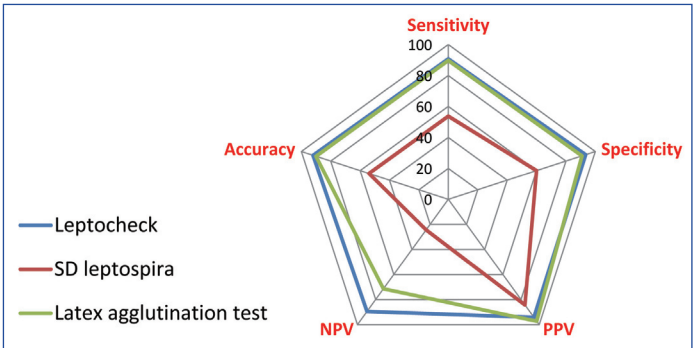
Rapid screening serological test which is sensitive early in the infection is needed. This is important because if treatment decisions are to be based on laboratory results, they must be made as early as possible, often without having available results from paired sera. When only samples from acutely ill patients were considered, the leptocheck WB and Latex agglutination test showed comparable sensitivity to the IgM-ELISA, whereas the sensitivity of the SD

Screening Test	%Sensitivity	%Specificity	%PPV	%NPV	%Accuracy
Leptocheck	90.7	93.4	94.2	89.5	92
Leptorapide	89.7	90.9	97.2	71.4	90
Sd Leptospira	53.7	60	84.3	24.4	54

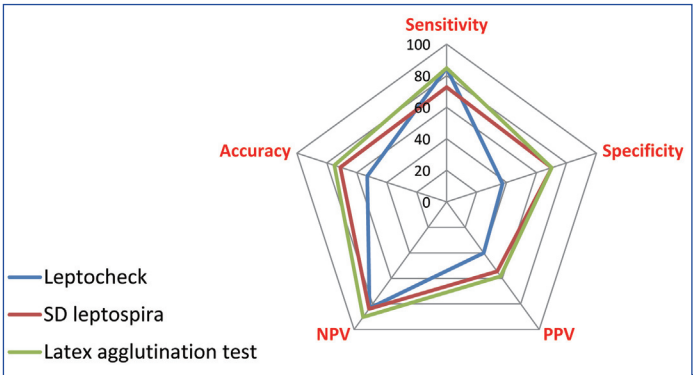
[Table/Fig-1]: Comparison of different screening (Rapid) tests considering ELISA as Gold standard.

Screening Test	%Sensitivity	%Specificity	%PPV	%NPV	%Accuracy
Leptocheck	84.8	37.3	40	83.3	53
Leptorapide	84.8	70.1	58.3	90.3	75
Sd Leptospira	72.7	70.1	54.5	83.9	71

[Table/Fig-2]: Comparison of different screening (Rapid) tests considering MAT as Gold standard.



[Table/Fig-3]: Comparision of Rapid tests considering ELISA as Gold std



[Table/Fig-4]: Comparision of Rapid tests considering MAT as Gold std

Leptospira IgM/IgG was closer to that of the MAT. Considering MAT as reference and compared with various rapid tests, SD leptospira shows higher sensitivity, specificity, PPV, NPV and accuracy values 72.7%, 70.1%, 54.5%, 83.9% and 71% respectively as both MAT and SD leptospira tests detects IgG antibodies appearing later during the course of disease. In S Shekatkar et al.,study showed the sensitivity and specificity of Latex agglutination test was 90.62% and 91.96% respectively compared to MAT (gold standard) which was slightly lower in our study that sensitivity and specificity of LAT was 84.8% and 70.1% respectively [13].

There are several possible explanations for the variability in screening test sensitivity observed between studies. The selection of the control population, which may cause difference [7]. The collection of healthy control sera was not done from endemic area, as cross reactivity also occurred in healthy controls, possibly as a result of preexisting condition. Lijmer et al., report that studies using a diseased population and a separate control group significantly overestimate the diagnostic performance of screening tests compared to studies using a single clinical population. The optimal design for assessing the accuracy of a diagnostic test is a prospective comparison of the "test and the reference test in a consecutive series of patients from a clinically relevant population [14]. Sensitivity of Leptospirosis screening tests may be affected by the prevalence of the various different infecting serogroups thereby effecting its performance. In all screening test for Leptospirosis diagnosis, antigen should be broadly reactive with different infecting Leptospira serovars. The characteristics of the Leptospiral antigen may differ from one place to another. So, the screening test should have ability to detect the antibodies produced against the site-specific leptospira serovars. Hence, laboratories need to validate the performance of screening tests in that particular setting in which they are to be used.

Sometimes, Leptospirosis patients might have co-infection or cross reactive antibodies of other diseases. Some of the control sera from other infectious etiology like syphilis, dengue, malaria, relapsing fever, lymes disease, legionellosis were not analyzed in this study as number of these disease agents have been reported by other investigators to cross react in leptospirosis serologic assay [3,5,15, 16]. In Stuart et al., study showed low sensitivity and specificity 47.3%, 75.5% of Leptotek IgM lateral flow test compared to gold standard MAT test [17], as MAT detects both IgM and IgG antibodies, it is difficult to differentiate between current clinical infection or past history of exposure to infection by doing only single MAT. At earlier stage of disease, genus specific IgM antibodies appear first so genus specific IgM immunoassay are expected to be positive than serovars specific MAT test. In this study, the specificity and sensitivity of latex-agglutination test (LAT) and Leptocheck WB showed comparable results to that genus specific IgM ELISA. LAT and Leptocheck WB have advantages of simple and rapid performance; and the use of stable antigens, which eliminates the necessity of maintaining live leptospiral cultures in diagnostic laboratories. The selection of a serodiagnostic assay is dependent on several factors, including the clinical likelihood of disease, the anticipated workload, and the availability of confirmatory testing in more specialized laboratories. Thus, in view of the reemerging zoonosis, the prompt diagnosis of Leptospirosis is essential for both patients care and efficient implementation of public health measures. It is therefore important to have an efficient diagnostic test that is rapid, accessible and practical to general physicians [18].

CONCLUSION

In this study, three rapid assays for early diagnosis of acute Leptospirosis in a hospital-based population were evaluated. Latex agglutination test kit and leptocheck WB were found to be highly sensitive and specific. Neither of these tests requires specialized equipment, and could be performed in peripheral laboratories with relatively little expertise. With either LA or leptocheck WB; human Leptospirosis will be diagnosed more readily and more accurately in the first week of fever for screening sera from acutely ill patients.

REFERENCES

[1] World Health Organization. Leptospirosis worldwide, 1999. Wkly. *Epidemiol Rec.* 1999;74:237-42.
[2] Bajani MD, DA Ashford, SL Bragg, CW Woods, T Aye, RA Spiegel, et al. Evaluation of Four Commercially Available Rapid Serologic Tests for Diagnosis of Leptospirosis. *J of Clinical Microbiol*. 2003;41(2):803–09.
[3] Levett PN, SL Branch, CU Whittington, CN Edwards, H Paxton. Two methods for rapid serological diagnosis of acute leptospirosis. *Clin Diagn Lab Immunol*. 2001;8:349-51.
[4] H Dupont, DD Perdrizet, JL Perie, SZ Hansen, B Jarrige, JB Daijardin. Leptospirosis: Prognostic Factor Associated with Mortality. *Clinical Infectious diseases*. 1997;25:720-24.
[5] Levett PN, Whittington CU. Evaluation of the indirect haemagglutination assay for diagnosis of acute leptospirosis. *J ClinMicrobiol*. 1998;36:11–14.
[6] P Naigowit, P Wangroongsarb, W Petkanchanapong, O Luepaktra, P Warachit. A comparative Evaluation of Different Methods for the Serological Diagnosis of Leptospirosis. *J Trop Med Parasitol*. 2000;23:59-65.
[7] VB Dohe, SS Pol, AP Karmarkar, RS Bharadwaj. Two Test Strategies for the Diagnosis of Leptospirosis. *Bombay Hospital*. 2009;51(1):18-21.
[8] Vijayachari P, Suganan AP, Sehgal SC. Role of microscopic agglutination test (MAT) as a diagnostic tool during acute stage of leptospirosisin low and high endemic areas. *Indian J Med Res*. 2001;114:99-106.
[9] Plank R, Dean D. Overview of the epidemiology, microbiology, and pathogenesis of Leptospira spp. In humans. *Microbes Infect*. 2000;2:1265-76.
[10] Goris MGA, Leeflang MMG, Loden M, Wagenaar JFP, Klatser PR, et al. Prospective Evaluation of Three Rapid Diagnostic Tests for Diagnosis of Human Leptospirosis. *PLoS Negl Trop Dis*. 2013;7(7):e2290. doi:10.1371/journal.pntd.0002290.
[11] Chiat Han Chang, Mehdi Riazi, Muhammad Hafiznur Yunus, Sabariah Osman, Rahmah Noordin. Limited diagnostic value of two commercial rapid tests for acute leptospirosis detection in Malaysia. *Diagnostic Microbiology and Infectious Disease*. 2014;80:278.
[12] Susannah Colt, Boris I Pavlin, Jacob L Kool, Leaser Johnson, Judith P McCool, Alistair J Woodward. Human leptospirosis in The Federated States of Micronesia: a hospital-based febrile illness survey. *BMC Infectious Diseases*. 2014;14:267.
[13] S Shekatkar, NS Achrya, BN Harish, SC Parija. Comparision of an in-house latex agglutination test with IgM ELISA and MAT in the diagnosis of leptospirosis. *Indian j of Medical Microbiology*. 2010;28(3):238-40.
[14] Lijmer JG, BW Mol, S Heisterkamp, GJ Bonsel, MH Prins, JHP van der Meulen, et al. Empirical evidence of design related bias in studies of diagnostic test. *JAMA*. 1999;282;1061-66.

[15] Cinco M, D Balanzin, E Banfi. Evaluation of an immunoenzymatic test (ELISA) for the diagnosis of leptospirosis in Italy. *Eur J Epidemiol*. 1992.8:677-82.
[16] Winslow WE, Merry DJ, Pirc ML. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. *J Clin Microbiol.* 1997;35:1938–42.
[17] Stuart D Blacksell, Lee Smythe, Rattanaphone Phetsouvanh, Michael Dohnt, Rudy Hartskeerl, Meegan Symonds, et al. Limited diagnostic capacities of two commercial assays for the detection of leptospira immunoglobulin M. Antibodies in Laos. *Clin Vaccine Immunol*. 2006;13(10):1166-69.
[18] WY Sekhar, EH Soo, V Gopalakrishnan, S Devi. Leptospirosis in Kuala Lumpur and the Comparative Evaluation of Two Rapid Commercial Diagnostic Kits Against the MAT Test for the Detection of Antibodies to LeptospiraInterrogans. *Singapore Med J*. 2000;41(8):370-75.

PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Microbiology, Government Medical College, Surat, India.
2. Assistant Professor, Department of Microbiology, Government Medical College, Surat, India.
3. Professor and Head, Department of Microbiology, Government Medical College, Surat, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Tanvi Panwala,
E-7, Vighneshwar Estate, Nanpura, Timaliavad, Surat-395001, India.
E-mail: drtanvi2006@yahoo.co.in

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Sep 10, 2014**
Date of Peer Review: **Dec 22, 2014**
Date of Acceptance: **Jan 06, 2015**
Date of Publishing: **Feb 01, 2015**

ABSTRACTS OF FREE PAPERS

B1 - A pilot study comparing two rapid immunodiagnostics tests with the microscopic agglutination test (MAT) for the detection of leptospira antibodies

Eugene EJ[1], Wickramasinghe SA[1], Kalugalage TL[1], Rodrigo C[2], Wickremesinghe H[3], Dikmadugoda N[3], Somaratne P[4] , De Silva HJ[5], Rajapakse S[2] , Handunnetti SM[1]

[1]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo
[2]Department of Clinical Medicine, Faculty of Medicine, University of Colombo
[3]Base Hospital, Homagama
[4]Department of Microbiology, Medical Research Institute, Colombo
[5]Department of Medicine, Faculty of Medicine, University of Kelaniya.

Background: In Sri Lanka, patients are often treated for leptospirosis based on a clinical diagnosis. Serological confirmation is usually not obtainable during the acute stage of the disease. There is a need for rapid immunodiagnostics to confirm leptospirosis infections, to influence the treatment and management of severe leptospirosis and for research – on immunopathogenesis. Rapid immunodiagnostic assays based on *e*nzyme *l*inked *i*mmunosorbent *a*ssay (ELISA) and immunochromatographic techniques are available to detect leptospira specific IgM antibodies which are prevalent during the early stages of acute infections.

Objective: To compare -two rapid immunodiagnostic tests, an IgM ELISA and the *L*epto*c*heck-WB *t*est (LCT) against the *m*icroscopic *a*gglutination *t*est (MAT) to determine their applicability.

Methods: A set of sera (n=83) collected in 2010, for which MAT titers were available, was used to perform the IgM ELISA and LCT. MAT ≥400 was used as the reference standard for a positive antibody test.

Results: Percentage positivity for IgM ELISA, LCT and MAT were 48.2%, 55.4% and 48.2% respectively. Both IgM ELISA and LCT detected acute infection by day 3 of illness. For LCT, the overall sensitivity, specificity, accuracy, PPV and NPV against MAT (86.5%, 75.0%, 79.6%, 69.6% and 89.4% respectively) were higher compared to the respective values for IgM ELISA (50.0%, 62.3%, 57.1%, 50.0%, 62.3%). The highest values of Accuracy, Specificity and PPV were observed during the first week for LCT and during the second week for IgM ELISA. The highest agreement of compatibility was observed between LCT and MAT≥400 (κ=0.568) and there was a good agreement between LCT and IgM ELISA (κ=0.520) using SPSS 17.

Conclusion: The high sensitivity and specificity, ease of use and no requirement of specialized skills and equipment, makes LCT a good choice for screening compared to MAT and its application needs to be to further investigated.

This work was supported by the IBMBB, University of Colombo.

PLOS ONE

RESEARCH ARTICLE

Diagnosis of Leptospirosis: Comparison between Microscopic Agglutination Test, IgM-ELISA and IgM Rapid Immunochromatography Test

Roshan Niloofa[1]*, Narmada Fernando[1], Nipun Lakshitha de Silva[2], Lilani Karunanayake[3], Hasith Wickramasinghe[4], Nandana Dikmadugoda[4], Gayani Premawansa[5], Rajitha Wickramasinghe[6], H. Janaka de Silva[6], Sunil Premawansa[7], Senaka Rajapakse[2], Shiroma Handunnetti[1]

1 Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo, Sri Lanka, **2** Tropical Medicine Research Unit, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka, **3** National Reference Laboratory for *Leptospira*, Medical Research Institute, Colombo, Sri Lanka, **4** Base Hospital Homagama, Homagama, Sri Lanka, **5** Colombo North Teaching Hospital, Ragama, Sri Lanka, **6** Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka, **7** Faculty of Science, University of Colombo, Colombo, Sri Lanka

* rosh86niloo@gmail.com

OPEN ACCESS

Citation: Niloofa R, Fernando N, de Silva NL, Karunanayake L, Wickramasinghe H, Dikmadugoda N, et al. (2015) Diagnosis of Leptospirosis: Comparison between Microscopic Agglutination Test, IgM-ELISA and IgM Rapid Immunochromatography Test. PLoS ONE 10(6): e0129236. doi:10.1371/journal.pone.0129236

Academic Editor: Odir A Dellagostin, Federal University of Pelotas, BRAZIL

Received: March 6, 2015

Accepted: May 6, 2015

Published: June 18, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Research grant funding from National Science Foundation, Sri Lanka was used, grant # rg/2011/hs/19. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Leptospirosis is diagnosed on clinical grounds, and confirmed by microscopic agglutination test (MAT). IgM-ELISA (Serion-Virion) and immunochromatography test (Leptocheck-WB) are two immunodiagnostic assays for leptospirosis. Their sensitivity, specificity and applicability in Sri Lanka have not been systematically evaluated.

Methods

Clinically diagnosed leptospirosis patients (n = 919) were recruited from three hospitals in the Western Province of Sri Lanka, during June 2012 to December 2013. MAT, IgM-ELISA and Leptocheck-WB were performed on all patient sera. MAT titer of $\geq$400 in single sample, four-fold rise or seroconversion $\geq$100 in paired samples were considered as positive for MAT. For diagnostic confirmation, MAT was performed during both acute and convalescent phases. Anti-leptospiral IgM $\geq$20 IU/ml and appearance of a band in the test window were considered as positive for IgM-ELISA and Leptocheck-WB test respectively. Patients with an alternative diagnosis (n = 31) were excluded. Data analysis was performed using two methods, i) considering MAT as reference standard and ii) using Bayesian latent class model analysis (BLCM) which considers each test as imperfect.

Results

MAT, IgM-ELISA and Leptocheck-WB positivity were 39.8%, 45.8% and 38.7% respectively during the acute phase. Acute-phase MAT had specificity and sensitivity of 95.7% and

55.3% respectively, when compared to overall MAT positivity. IgM-ELISA and Leptocheck-WB had similar diagnostic sensitivity when compared with acute-phase MAT as the gold standard, although IgM-ELISA showed higher specificity (84.5%) than Leptocheck-WB (73.3%). BLCM analysis showed that IgM-ELISA and Leptocheck-WB had similar sensitivities (86.0% and 87.4%), while acute-phase MAT had the lowest sensitivity (77.4%). However, acute-phase MAT had high specificity (97.6%), while IgM-ELISA and Leptocheck-WB showed similar but lower specificity (84.5% and 82.9%).

Conclusions

Both IgM-ELISA and Leptocheck-WB shows similar sensitivities and specificities. IgM-ELISA may be superior to MAT during the acute phase and suitable for early diagnosis of leptospirosis. Leptocheck-WB is suitable as a rapid immunodiagnostic screening test for resource limited settings.

Introduction

Leptospirosis is a globally widespread zoonosis caused by pathogenic spirochetes belonging to the genus *Leptospira*[1]. An estimated 500,000 cases occur annually, with fatality range rising up to 70% in different cohorts[2]. Leptospirosis is endemic to Sri Lanka, with outbreaks occurring every four to five years. A large outbreak took place in 2008, with 7406 reported cases and 204 deaths, giving an incidence rate of 35.7 per 100,000 populations, and case fatality rate of 2.75%[3].

Human hosts commonly acquire infection through skin abrasions and mucosal surfaces following contact with water or soil contaminated with urine of infected rodents or other mammals. Leptospirosis has a wide range of clinical manifestations, from a simple febrile illness to a severe and potentially fatal illness characterized by acute kidney injury, liver derangement, pulmonary haemorrhage, bleeding, and cardiac involvement. In most clinical settings, there is limited availability of specific diagnostic tests, and treating physicians often rely on clinical features to make a probable diagnosis of leptospirosis. This is indeed a problem in areas of high incidence of other infections with similar clinical picture, such as dengue, rickettsial infection, malaria and hantavirus infections[4].

Laboratory diagnosis of leptospirosis is based on several methods: the microscopic agglutination test (MAT), detection of organism DNA by polymerase chain reaction (PCR), isolation of the organism through culture methods, or detection of antibodies to the organism[5]. Isolation of *Leptospira* spp. from clinical samples has low diagnostic sensitivity, requires specialized expertise, and most importantly takes too long to be of use to the treating team[6]. Antigens can be detected by histological, histochemical or immunestaining techniques and *Leptospira* DNA by PCR. Unfortunately, none of these tests are currently suitable for routine laboratory use, because of technical limitations and low sensitivity[5]. MAT is considered the reference immunological test, and detects both immunoglobulin M (IgM) and immunoglobulin G (IgG) class agglutinating antibodies. However, this test requires a high level of technical expertise, and the maintenance of a large panel of live pathogenic *Leptospira* standard cultures. The use of live *Leptospira* organisms also creates a risk of laboratory acquired infection to the laboratory technicians[7]. MAT also gives large number of false negative results in the early course of infection, as IgM antibodies detectable by MAT appear after day 8 of the illness, reach the peak

by the fourth week, and furthermore, detectable titers of serovar specific functional antibodies may persist for several months[8–10]. MAT requires testing paired sera collected at appropriate time intervals for an accurate interpretation of results. Thus, while it is of value for epidemiological purposes, there are limitations in its value in the acute clinical setting. Currently, MAT is routinely available only in a central reference laboratory in Sri Lanka, i.e., the National Reference Laboratory for *Leptospira*, Medical Research Institute (MRI), Colombo[11]. At the time of conducting this study, only *Leptospira biflexa* serovar Patoc strain Patoc I was used by the MRI.

There is thus a clear need for reliable and valid rapid diagnostic tests for leptospirosis which can be made available to clinicians, in order to diagnose and treat leptospirosis during early course of infection. The ideal diagnostic test for leptospirosis should have high sensitivity and specificity during the acute phase, be widely available at reasonable cost, and give quick results. Several other immunodiagnostics have been evaluated as alternatives to MAT, such as Ig M detectable enzyme linked immune sorbent assay (IgM-ELISA), dot ELISA, indirect hemagglutination assay (IHA), immunofluorescence assay (IFA), *Leptospira* dipstick test and *Leptospira* immunochromatography test[12–14]. While these are relatively easier to perform when compared with MAT, their diagnostic accuracies have not been fully established. IgM-ELISA shows promise as an alternative to MAT, as many laboratories in tropical countries have facilities to perform the test[15, 16]. Some studies have reported that IgM-ELISA has high sensitivity and specificity[15, 17]. However, one study has been reported from Sri Lanka evaluating a commercially available immunodiagnostic ELISA (InstitutVirion\Serion GmbH, Warburg, Germany) kit showing very low sensitivity and specificity[18]. In this study, the acute phase IgM-ELISA was compared with diagnostic confirmation based on a four-fold rise in titer between acute and convalescent samples, and not against the immunological reference standard MAT. Leptocheck-WB test is a commercially available immunochromatographic test which identifies IgM, does not require any specialized laboratory facilities, and provides results within 15 minutes [13]. Leptocheck-WB has been evaluated in limited studies.

Although MAT is usually considered the immunological 'gold' standard for diagnosis, as mentioned above, MAT has inherent flaws. There has been much debate about the validity of using MAT as an immunological gold standard for evaluation of rapid diagnostics[19]. Bayesian latent class modelling, a statistical model which assumes that all tests are imperfect, has been suggested as a more suitable method for evaluating diagnostic tests, including immunodiagnostics for leptospirosis[19–21].

In this study, we evaluated two commercially available tests detecting *L. biflexa* serovar Patoc strain Patoc I specific IgM antibodies, and MAT detecting both agglutinating IgM and IgG antibodies against only *L. biflexa* serovar Patoc strain Patoc I. We analyzed our findings using two statistical models, i.e., taking MAT as the gold standard, and Bayesian latent class modelling.

Methods

The Standards for the Reporting of Diagnostic Accuracy Testing (STARD) were adhered to in this study (S1 Checklist)[22].

Study population

A total of 919 patients were enrolled in this study, from three hospitals in the Western Province of Sri Lanka. The Western Province is the most highly populated province in the country, with a square area of 3709 km^2 and population of 5.72 million[23]. An analysis of hospital based sentinel surveillance data of leptospirosis over 4 years in Sri Lanka has confirmed that of nearly

4000 suspected cases, 47% were from this province[24]. The three Hospitals were the National Hospital of Sri Lanka (NHSL), Colombo North Teaching Hospital (CNTH) and Base Hospital Homagama (BHH). Patients were recruited from June 2012 to May 2014. Patients over the age of 12 years, with a suspected diagnosis of Leptospirosis, admitted to the medical wards of these hospitals were enrolled. A suspected diagnosis of leptospirosis was defined based on the WHO-LERG epidemiological criteria[25], i.e., acute febrile illness with headache, myalgia, arthralgia, conjuctival suffusion, meningeal irritation, anuria, oliguria, protreinuria, jaundice, hemorrhages, cardiac arrhythmia or skin rash, or a contact history of exposure to water or soil contaminated with urine of infected animals. Patients with a definitive alternative diagnosis on presentation, such as dengue, pneumonia, meningitis, or other bacterial sepsis, and pregnant women were excluded from the study. Data was collected by investigators who were not directly involved in patient care. Demographic and clinical data and laboratory and other investigation findings were collected until the point of discharge or death, using a structured interviewer administered questionnaire.

Laboratory Methods

Five milliliters of blood were collected by sterile venepuncture and allowed to clot at 37°C, and serum was separated by centrifugation at 800 *g* for 10 minutes. Leptocheck-WB and MAT were performed immediately after recruitment. Sera were stored at -20°C until the performance of IgM-ELISA. All enrolled patients who survived were requested to return for convalescent sampling on day 21 from disease onset, during which 2 mL of blood taken for convalescent MAT.

Microscopic agglutination test. MAT was performed at the Reference Laboratory for Leptospirosis, Medical Research Institute, Colombo employing standard procedure[26]. Live organisms of *L.bilfexa* serovar Patoc strain Patoc I were cultured and maintained in EMJH (Ellinghausen- McCullough-Jonson-Harris) liquid media at room temperature. Serially diluted from the dilution of 1:100, serum specimens were added to the live *Leptospira* cell suspension in 96well round bottomed microtiter plates, and incubated for two hours at 37°C. Agglutination was examined under a magnification of 20X using dark field microscopy. The reciprocal of the highest dilution agglutinating at least 50% of the *Leptospira* organisms, was considered as the reporting titer. Single acute MAT positivity was defined as a titer of $\geq$400. Final MAT positivity was defined as a titer of $\geq$400 in single sample, sero-conversion from negative to a titer $\geq$100 or a four-fold rise in titer in paired (acute and convalescent) samples[25, 27].

Immunochromatography test. Leptocheck-WB (Zephyr Biomedicals, India) test was performed according to manufacturer's instructions[28]with a small modification. Five drops of running buffer were added following the addition of 20 μL serum to the test window. Although the manufacturer's instructions state that 10 μL of serum should be added, we performed a preliminary study with a small number of samples using both 10 μL and 20 μL of serum which demonstrated that the positive bands were persistent with 20 μL of serum without altering the actual result. Results were read visually after 15 minutes of incubation at room temperature. Anti-human IgM colloidal gold conjugate forms a complex with IgM antibodies in the sample while it flows through the membrane assembly of the test device. Antigens from *L. biflexa* serovar Patoc strain Patoc I are coated on the window 'T' of membrane capture, and immobilize the antibody-conjugate complex if present in the sample. This forms a red color band at the test region 'T'. The un-reacted conjugate and the unbound complex, if any, along with rabbit globulin colloidal gold conjugate move further on the membrane and are subsequently immobilized by the anti-rabbit antiserum coated at the control region 'C' of the membrane assembly, forming a red color band. Presence of bands in the test and control windows was read as positive, while absence of a band in the test window with the presence of control band was read as

negative. Absence of a band in the control window was read as invalid test and test was repeated.

IgM-ELISA. IgM-ELISA (InstitutVirion\Serion GmbH, Warburg, Germany) was performed according to manufacturer's instructions[29]. Briefly, rheumatoid factor (RF) absorbent was diluted 1:4 in dilution buffer to obtain RF dilution buffer. This ELISA uses crude antigens from an isolated, concentrated and partially purified extract of *L. biflexa* serovar Patoc strain Patoc I, which contains genus specific epitopes for all *Leptospira* serovars. Sera sample was diluted 1:100 in RF dilution buffer and incubated for 15 minutes at room temperature. This is performed for the removal of IgM rheumatoid factors. Standards and diluted samples were transferred to the microtiter wells and incubated at 37°C for 60 minutes in a moist chamber. Residual serum was removed from the wells by washing four times with the wash buffer; anti-human IgM conjugated to alkaline phosphatase was added and incubated at 37°C for 30 minutes in a moist chamber. Wells were washed four times with the wash buffer; substrate *p*-nitrophenyl phosphate was added and incubated at 37°C for 30 minutes in a moist chamber. Sodium hydroxide was added and the enzyme substrate reaction was stopped for the readings. Optical density against the substrate blank was read at 405 nm and at a background of wavelength of 650 nm. Each kit was performed with a negative control, positive control and cut-off calibrator (standards) in duplicate. Absorbance reading of the above in a test obeying the specifications of the Serion ELISA indicates that the test is valid. Results were obtained using the evaluation table provided along with the kit. Interpretation of results for Serion ELISA classic *Leptospira* IgM was as follows: anti-leptospiral IgM $<$15 IU/ml gives a negative result suggesting no evidence of a recent infection, 15–20 IU/ml gives a borderline result suggesting that may be a recent infection and $\geq$20 IU/ml gives a positive result which is interpreted as a recent or current infection.

All sera with a positive result for any of the above tests were tested for hantaviral infection, using a commercially available IgM-ELISA kit (InstitutVirion\SerionGmgH, Warburg, Germany). The assay was performed according to the manufacturer's instructions[30]. Results were obtained using the evaluation table provided along with the kit. This provided quantities of anti-hantaviral IgM in IU per mL and qualitative results: negative ($<$10 IU/mL) result suggesting no evidence of recent infection, borderline (10 to 15 IU/mL) result suggesting possible recent infection, and positive ($\geq$15 IU/mL) result suggesting a recent or current infection. Borderline results of both ELISAs were considered as negatives. Hantaviral IgM positives were excluded from the analysis.

Ethics approval

Ethics approval was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Colombo (EC-12-056). Patients were recruited to the study after obtaining informed written consent from the patient, next of kin or care-takers when patients were severe. Informed written consent was obtained from parents or guardian on behalf of patients aged below 18 years.

Statistical analyses

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 17.0. We considered positive MAT under two circumstances: a) MAT during the acute phase of illness, a titer of $\geq$400 (Acute MAT), and b) either acute MAT, or a four-fold rise in MAT titer between acute and convalescent samples, or seroconversion on MAT to a titer of $\geq$100 (Final MAT). Patients positive on 'Final MAT' were considered true positives for leptospirosis for the purpose of gold standard analysis. First, the diagnostic accuracy of 'acute MAT'

was evaluated with 'final MAT' as gold standard, where data was available. Next, sensitivities, specificities, positive and negative predictive values of Leptocheck-WB and IgM-ELISA were calculated with the 'final MAT' as the gold standard. Finally we compared both 'Acute MAT' and 'Final MAT' separately with IgM-ELISA and Leptocheck-WB using Bayesian latent class modelling. The MICE tool (Modelling for Infectious Disease Centre, Mahidol-Oxford Research Unit)[31, 32] was used to perform Bayesian latent class modelling.

Results

We enrolled a total of 919 patients with acute fever and a suspected diagnosis of leptospirosis (NHSL-689, BHH -165, CNTH -34). Of these, 31 patients were excluded from the analysis as they were diagnosed as having dengue, typhoid fever, and sepsis or hantaviral infection. Data of 888 patients were included in the final analysis. The male to female ratio was 9:1. Mean age was 42 years (SD±16). Samples were collected at median of 6 days (SD±3.58) after the onset of symptoms. Follow-up samples were received from 255/888 patients. The baseline characteristics of the patients are shown in Table 1. Further details about participants and diagnostic assays are shown in Fig 1.

Positivity based on MAT

Based on the criteria considered as MAT positivity (i.e., either titer of ≥400 in single sample, or seroconversion from negative to a titer ≥100, or a four-fold rise in titer in paired samples), a total of 354 (39.8%) patients were MAT positive, out of the total of 888 patients included in the final analysis. Of these, 293 patients had a single MAT positive, and another 61 patients were positive based on paired MAT.

Table 1. Baseline demographic and clinical profile of enrolled patients.

Characteristic		Baseline data
Age, Mean ±SD; (Range)		41.7 ±15.6; (13–80)
Male: Female Ratio		9:1
Exposure to contaminated water		
	Yes	597
	No	256
Occupation		
	Farming	119
	Other	614
	Unemployed	120
Fever		888
Headach		760
Myalgia		778
Nausea and vomiting		459
Conjuctival suffusion		416
Jaundice		196
Acute kidney injury		304
Hemorrhage		225
Lung involvement		12
ICU admissions		35
Received haemodialysis		139
Deaths		26

doi:10.1371/journal.pone.0129236.t001

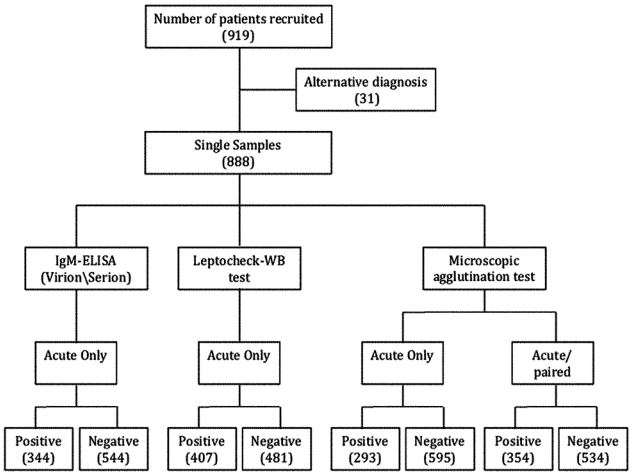


Fig 1. Flowchart showing the participants and the results of leptospirosis diagnostic tests microscopic agglutination test (MAT), Leptocheck-WB and IgM-ELISA.

doi:10.1371/journal.pone.0129236.g001

Accuracy of single acute MAT

Using the subset of patients who had both acute and convalescent samples analyzed (n = 255), we compared the accuracy of a single MAT performed during the acute phase of illness (defined as **Acute MAT**), against **Final MAT** (i.e., positivity or negativity based on any of the three MAT criteria). In this cohort, 93 were MAT positive in the acute phase, and 161 were positive for when convalescent samples were considered (Table 2). Acute MAT had a sensitivity of 55.3%, specificity of 95.7%, a positive predictive value (PPV) of 0.95 and a negative predictive value (NPV) of 0.55. While MAT is a highly specific test, it lacks sensitivity during the acute stage of infection.

IgM-ELISA and Leptocheck-WB compared with MAT positivity as gold standard

Using a single acute MAT (Acute MAT) as a reference standard, 33% of patients in the cohort had confirmed leptospirosis. Leptocheck-WB had a sensitivity of 84.6% while IgM-ELISA had a sensitivity of 86.0% (Table 3) (S1 and S2 Tables); there was no significant difference in sensitivity between the 2 methods. The specificity of IgM-ELISA [84.5% (81.3%-87.3%)] was significantly higher than that of Leptocheck-WB [73.3% (69.5%-76.8%)]. When a combination of acute samples and paired samples for MAT (i.e., Final MAT) were considered, the proportion of confirmed leptospirosis increased to 43.4% (39.5%-47.5%). There was a significant reduction in the sensitivity of leptocheck-WB test. However, IgM-ELISA retained good levels of sensitivity or specificity.

Table 2. MAT during the acute phase compared with overall MAT positivity.

MAT Test	Positive	Negative	Sensitivity (%)	Specificity (%)
Acute MAT	93	162	55.3	95.7
Final MAT	161	94	100	100

Acute MAT defined as MAT performed on acute serum sample. Final MAT defined as positivity or negativity based on acute MAT, acute and convalescent samples, or seroconversion, and used as the reference standard. Positive and negative values are given as absolute numbers.

doi:10.1371/journal.pone.0129236.t002

Table 3. Prevalence, sensitivities, specificities and positive and negative predictive values of Leptocheck-WB and IgM-ELISA using the MAT as gold standard and Bayesian latent class models.

	MAT as gold standard (%)*		Bayesian latent class model (%)+	
Parameters	**Acute only**	**Acute or Paired**	**Acute only**	**Acute or Paired**
Prevalence	33.0(29.9–36.2)	43.4 (39.5–47.5)	40.8 (37.0–44.9)	43.4 (39.5–47.5)
MAT				
Sensitivity	100	100	77.4 (71.8–82.3)	85.4 (80.6–89.6)
Specificity	100	100	97.6 (95.3–99.2)	94.3 (91.2–96.8)
PPV	100	100	95.6 (91.7–98.6)	92.0 (87.6–95.7)
NPV	100	100	86.2 (82.0–89.6)	89.4 (85.3–92.6)
Leptocheck-WB Test				
Sensitivity	84.6 (79.9–88.5)	80.8 (76.2–84.7)	87.4 (83.0–91.3)	86.2 (81.5–90.0)
Specificity	73.3 (69.5–76.8)	76.9 (73.0–80.4)	82.9 (79.1–86.1)	84.3 (80.3–87.7)
PPV	60.9 (56.0–65.7)	70.3 (65.5–74.6)	77.8 (72.9–82.4)	80.8 (75.8–85.1)
NPV	90.6 (87.6–93.0)	85.6 (82.0–88.5)	90.5 (86.6–93.5)	88.8 (84.6–92.2)
IgM-ELISA (Virion\Serion)				
Sensitivity	86.0 (81.4–89.7)	80.2 (75.6–84.2)	86.0 (81.4–89.7)	86.9 (82.2–91.0)
Specificity	84.5 (81.3–87.3)	88.5 (85.4–91.1)	84.5 (81.3–87.3)	97.5 (95.1–99.7)
PPV	73.3 (68.2–77.8)	82.6 (78.0–86.3)	73.3 (68.2–77.8)	96.4 (92.5–99.5)
NPV	92.5 (89.8–94.5)	86.9 (83.7–89.6)	92.5 (89.8–94.5)	90.6 (86.9–93.7)

*Gold standard model assumed that MAT is perfect (100% sensitivity and 100% specificity; all patients with gold standard test positive are diseased and all patients with gold standard test negative are non-diseased). MAT titer $\geq$ 400 was considered to be positive. Values shown are estimated means with 95% confidence interval.

+Bayesian latent class model assumed that all tests evaluated are imperfect. Values shown are estimated median with 95% credible interval.

doi:10.1371/journal.pone.0129236.t003

Bayesian latent class modelling for MAT, IgM-ELISA and Leptocheck-WB test

Based on the proportion of patients diagnosed with leptospirosis among this group of patients being 0.41 (0.37–0.45), and using only acute samples (i.e., acute MAT), sensitivities of MAT, Leptocheck-WB and IgM-ELISA were 77.4% (71.8%-82.3%), 87.4% (83.0%-91.3%) and 86.0% (81.4%-89.7%), respectively, and specificities were 97.6% (95.6%-99.2%), 82.9% (79.1%-86.1%) and 84.5% (81.3%-87.3%), respectively.

The proportion of patients diagnosed with leptospirosis among this group of patients using both acute and paired samples was 0.43 (0.39–0.47). Sensitivities of MAT, Leptocheck-WB and IgM-ELISA were 85.4% (80.6%-89.6%), 86.2% (81.5%-90.0%) and 86.9% (82.2%-90.0%) respectively; the specificities were 94.3% (91.2%-96.8%), 84.3% (80.3%-87.7%) and 97.5% (95.1%-99.7%) respectively.

Discussion

Early and definitive diagnosis of leptospirosis is important to guide the clinician to commence appropriate treatment, and prioritize resource allocation for management of complications. Although MAT is generally considered the immunological gold standard, our analysis shows that MAT has poor sensitivity when performed early; the use of both acute and convalescent samples increases the sensitivity of MAT as a test to diagnose leptospirosis. Bayesian latent class modelling also demonstrated that the sensitivity of MAT was relatively low, but increased when considering both acute and convalescent samples. Historically, MAT is used as the

reference standard for the serological assays and widely used for the confirmation of the disease. However, our study suggests that MAT is an imperfect gold standard for the early detection of leptospirosis. MAT detects agglutinating antibodies of both IgM and IgG classes. These functional antibodies take 1–2 days longer than the appearance of *Leptospira* genus specific IgM antibodies. The period for which IgM and IgG antibodies detected by MAT persist following acute infection is a subject of controversy. Infection with certain types of serovars, have been shown to produce longer lasting immunity, such as the Autumnalis serogroup[10]. Nonetheless our study showed high specificity with acute MAT. HoweThus, MAT is useful as a confirmatory test, and for epidemiological purposes.

In our study, the Patoc-1 genus specific strain was used in all three tests (MAT, Leptocheck-WB and IgM-ELISA) that were evaluated. As discussed elsewhere, genus specific antibodies appear earlier than serovar specific antibodies. So at the acute stage of infection, genus specific tests, especially IgM detecting assays are expected to give positive results while serovar specific tests are still not able to detect the antibodies.

The gold standard analysis of our study was compared with the other studies (Table 4). In previous studies, Serion IgM-ELISA's sensitivity ranges from 48% to 100% and specificity ranges from 88.6% to 98%. Leptocheck-WB test's sensitivity ranges from 78 to 93.81% and specificity ranges from 86.81 to 98%. These results show a correlation with the results of our present study.

High sensitivity and specificity of IgM-ELISA during the acute phase of illness using single sample, make *Leptospira* genus specific IgM detecting ELISA suitable for both early as well as definitive diagnosis. This test also gives high PPV and NPV during the early phase of infection.

Leptocheck-WB also has a high sensitivity and reasonable specificity. It is easy to perform, rapid method that takes only 15–20 minutes, and does not require any special equipment. In comparison, IgM-ELISA has several steps in its procedure, requires a technically skilled person, takes about 4 hours to perform, and requires an ELISA plate reader. Leptocheck-WB test gives consistent results, and the deep color bands, which are stable for more than 12 months. Kit contents are stable and can be transported and stored at ambient temperatures, and are small, portable packages. In our study, the approximate cost per specimen for IgM-ELISA was US $ 3.4 whereas Leptocheck-WB cost was only approximately US$ 1.9. The higher sensitivity and NPV of Leptocheck-WB, together with its lower cost and ease of use, suggests that it would be useful as a screening test. The higher specificity, sensitivity, PPV and NPV of IgM-ELISA suggest that IgM-ELISA is appropriate for confirmation and definitive diagnosis, and may be superior to MAT, especially during the acute phase of illness.

One limitation of our study was the use of *L. biflexa* serovar Patoc strain Patoc I as the base for all three diagnostic tests. At the time of conducting this study, this was the only strain for which MAT was available in the reference laboratory in Sri Lanka. Our future studies will incorporate testing against a panel of serovars.

Table 4. Results of the study in comparison with other studies.

	Reference	Sample size	Sensitivity	Specificity
IgM-ELISA	Panwala et al [13]	130	93.8	90.1
(Virion\Serion)	Kucerova et al [33]	45	100.0	88.6
	Effler et al [34]	344	48.0	98.0
	Present study	888	86.9	97.5
Leptocheck WB test	T Panwala[13]	130	93.8	86.8
	MG Goris[35]	197	78.0	98.0
	Present study	888	86.1	84.5

doi:10.1371/journal.pone.0129236.t004

Conclusion

MAT is an imperfect gold standard serological test for early diagnosis; its high specificity makes it a useful tool for confirmatory diagnosis, however it lacks sensitivity for use in diagnosis of acute illness. MAT would be an important tool for epidemiological purposes, such as identification of infecting serovars, and also to identify the prevalent serovar during an outbreak. IgM-ELISA (InstitutVirion\SerionGmgH, Warburg, Germany) is suitable for early and definitive diagnosis of acute leptospirosis. Leptocheck-WB test is suitable as a screening test for use in resource-limited settings. Our results reiterate the importance of proper evaluation of serological diagnostics[19] using statistical models that assume that all tests are imperfect.

Supporting Information

S1 Checklist. STARD checklist for reporting studies of diagnostic accuracy.
(DOC)

S1 Table. Results of three diagnostic tests on acute sample (n = 888).
(DOCX)

S2 Table. Results of three diagnostic tests on acute/paired sample (n = 888) used for MAT.
(DOCX)

Acknowledgments

We thank the consultants and ward staff of the National Hospital of Sri Lanka, Base Hospital Homagama and Colombo North Teaching Hospital, Sri Lanka; Laboratory staff of the National Reference Laboratory for Leptospira, Medical Research Institute, Sri Lanka; Dr Tharanga Fernando and Dr Sachith Maduranga for their support in recruitment of study subjects.

Author Contributions

Conceived and designed the experiments: SH SR SP HJdS RN. Performed the experiments: RN LK. Analyzed the data: RN SR RW. Contributed reagents/materials/analysis tools: RN NF NLdS HW ND GP. Wrote the paper: RN SR. Additional manuscript editing and correction: NLdS NF LK RW HJdS SP SH.

References

1. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. The Lancet Infectious Diseases. 2003; 3(12):757–71. doi: 10.1016/s1473-3099(03)00830-2 PMID: 14652202
2. Vijayachari P, Sugunan AP, Shriram AN. Leptospirosis: an emerging global public health problem. Journal of biosciences. 2008; 33(4):557–69. PMID: 19208981.
3. Agampodi SB, Peacock SJ, Thevanesam V, Nugegoda DB, Smythe L, Thaipadungpanit J, et al. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. The American journal of tropical medicine and hygiene. 2011; 85(3):471–8. doi: 10.4269/ajtmh.2011.11–0276 PMID: 21896807; PubMed Central PMCID: PMC3163869.
4. Izurieta R, Galwankar S, Clem A. Leptospirosis: The "mysterious" mimic. Journal of emergencies, trauma, and shock. 2008; 1(1):21–33. doi: 10.4103/0974-2700.40573 PMID: 19561939; PubMed Central PMCID: PMC2700559.
5. Musso D LSB. Laboratory diagnosis of leptospirosis: A challenge. Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi. 2013; 46(4):245–52. doi: 10.1016/j.jmii.2013.03.001 PMID: 23639380.
6. Wuthiekanun V, Chierakul W, Limmathurotsakul D, Smythe LD, Symonds ML, Dohnt MF, et al. Optimization of culture of Leptospira from humans with leptospirosis. Journal of clinical microbiology. 2007; 45 (4):1363–5. doi: 10.1128/JCM.02430-06 PMID: 17301285; PubMed Central PMCID: PMC1865830.

7. Sugunan AP, Natarajaseenivasan K, Vijayachari P, Sehgal SC. Percutaneous exposure resulting in laboratory-acquired leptospirosis—a case report. Journal of medical microbiology. 2004; 53(Pt 12):1259–62. doi: 10.1099/jmm.0.45735–0 PMID: 15585507.
8. Alder B FS. The antibodies involved in the human immune response to leptospiral infection. Journal of medical microbiology. 1978; 11:387–400. PMID: 722781
9. Cumberland P, Everard CO, Levett PN. Assessment of the efficacy of an IgM-elisa and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. The American journal of tropical medicine and hygiene. 1999; 61(5):731–4. PMID: 10586903.
10. Cumberland P, Everard CO, Wheeler JG, Levett PN. Persistence of anti-leptospiral IgM, IgG and agglutinating antibodies in patients presenting with acute febrile illness in Barbados 1979–1989. European journal of epidemiology. 2001; 17(7):601–8. PMID: 12086073.
11. Agampodi SB, Nugegoda DB, Thevanesam V. Determinants of leptospirosis in Sri Lanka: study protocol. BMC infectious diseases. 2010; 10:332. doi: 10.1186/1471-2334-10-332 PMID: 21087520; PubMed Central PMCID: PMC2994874.
12. Levett PN BS, Whittington CU, Edwards CN, Paxton H. Two methods for rapid serological diagnosis of acute leptospirosis. Clinical and diagnostic laboratory immunology. 2001; 8(2):349–51. doi: 10.1128/CDLI.8.2.349–351.2001 PMID: 11238220; PubMed Central PMCID: PMC96061.
13. Panwala T MS, Patel P. Seroprevalance of leptospirosis in south gujarat region by eveluating the two rapid commercial diagnostic kits against the MAT test for detection of antibodies to leptospira interrogans. National Journal of Community Medicine. 2011; 2(1):64–70.
14. Sehgal S, Vijayachari P, Sharma S, Sugunan A. Lepto dipstick a rapid and simple method for serodiagnosis of acute leptospirosis. Transactions of the royal society of tropical medicine and hygiene. 1999; 93:4. PMID: 10492776
15. Ooteman MC, Vago AR, Koury MC. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. Journal of microbiological methods. 2006; 65(2):247–57. doi: 10.1016/j.mimet.2005.07.015 PMID: 16253361.
16. Bajani MD, Ashford DA, Bragg SL, Woods CW, Aye T, Spiegel RA, et al. Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. Journal of clinical microbiology. 2003; 41(2):803–9. PMID: 12574287; PubMed Central PMCID: PMC149700.
17. Desakorn V, Wuthiekanun V, Thanachartwet V, Sahassananda D, Chierakul W, Apiwattanaporn A, et al. Accuracy of a commercial IgM ELISA for the diagnosis of human leptospirosis in Thailand. The American journal of tropical medicine and hygiene. 2012; 86(3):524–7. doi: 10.4269/ajtmh.2012.11–0423 PMID: 22403329; PubMed Central PMCID: PMC3284374.
18. Reller ME, Bodinayake C, Nagahawatte A, Devasiri V, Kodikara-Arachichi W, Strouse JJ, et al. Leptospirosis as frequent cause of acute febrile illness in southern Sri Lanka. Emerging infectious diseases. 2011; 17(9):1678–84. doi: 10.3201/eid1709.100915 PMID: 21888794; PubMed Central PMCID: PMC3322050.
19. 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. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2012; 55(3):322–31. doi: 10.1093/cid/cis403 PMID: 22523263; PubMed Central PMCID: PMC3393707.
20. Speybroeck N, Praet N, Claes F, Van Hong N, Torres K, Mao S, et al. True versus apparent malaria infection prevalence: the contribution of a Bayesian approach. PloS one. 2011; 6(2):e16705. doi: 10.1371/journal.pone.0016705 PMID: 21364745; PubMed Central PMCID: PMC3041757.
21. Limmathurotsakul D, Jamsen K, Arayawichanont A, Simpson JA, White LJ, Lee SJ, et al. Defining the true sensitivity of culture for the diagnosis of melioidosis using Bayesian latent class models. PloS one. 2010; 5(8):e12485. doi: 10.1371/journal.pone.0012485 PMID: 20830194; PubMed Central PMCID: PMC2932979.
22. Patrick M Bossuyt JBR, David E Bruns, Constantine A Gatsonis, Paul P Glasziou, Les M Irwig JGL, David Moher, Drummond Rennie, Henrica C W de Vet. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD iniative. British Medical Journal. 2003; 326:41–4. PMID: 12511463
23. Western Provincial Council Official Website 2013 [31st October 2014]. Available from: http://www.wpc.gov.lk.
24. Gamage CD, Amarasekera J, Palihawadana P, Samaraweera S, Mendis D, Janakan N, et al. Analysis of hospital-based sentinel surveillance data on leptospirosis in Sri Lanka, 2005–2008. Japanese journal of infectious diseases. 2012; 65(2):157–61. PMID: 22446124.
25. Organization WH. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group. 2011.

26. John R Cole CRSa ARP. Improved microtechnique for the Leptospiral microscopic agglutination test. Applied Microbiology. 1973; 25(6):976–80. PMID: 4736794

27. India WHOCof. Leptospirosis Laboratory Manual. 2007.

28. Rapid immunochromatographic test for IgM antibodies to Leptospirosis in human serum, plasma and whole blood Instructions Zephyr Biomedicals; 2013 [13th October 2013]. Available from: http://www.tulipgroup.com.

29. Virion/Serion. Serion ELISA classic Leptospira IgM (quantitatitive) instructions 2013 [7th November 2013]. Available from: http://www.virion-serion.de/fileadmin/templates/tpl1/global/download/flyer/Flyer_ELISA_classic_Leptospira__EN.pdf.

30. Virion/Serion. Serion ELISA classic Hanta virus puumula IgM (quantitatitive) instructions 2013 [7th November 2013]. Available from: http://www.virion-serion.de/fileadmin/templates/tpl1/global/download/flyer/Flyer_ELISA_classic_Hantavirus_Puumala__EN.pdf.

31. Lim C, Wannapinij P, White L, Day NP, Cooper BS, Peacock SJ, et al. Using a web-based application to define the accuracy of diagnostic tests when the gold standard is imperfect. PloS one. 2013; 8(11): e79489. doi: 10.1371/journal.pone.0079489 PMID: 24265775; PubMed Central PMCID: PMC3827152.

32. Modelling for Infectious disease CEnter. 2013.

33. Kucerova P, Cermakova Z, Pliskova L, Valenta Z, Pavlis O, Kubickova P. [Comparison of results of two serological methods for diagnosing leptospirosis—microagglutination test and ELISA]. Klinicka mikrobiologie a infekcni lekarstvi. 2011; 17(5):173–8. PMID: 22161754.

34. Effler PV, Bogard AK, Domen HY, Katz AR, Higa HY, Sasaki DM. Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. Journal of clinical microbiology. 2002; 40(4):1464–9. PMID: 11923374; PubMed Central PMCID: PMC140343.

35. Goris MG, Leeflang MM, Loden M, Wagenaar JF, Klatser PR, Hartskeerl RA, et al. Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. PLoS neglected tropical diseases. 2013; 7(7):e2290. doi: 10.1371/journal.pntd.0002290 PMID: 23875034; PubMed Central PMCID: PMC3708816.

Journal of Infection and Public Health 12 (2019) 263–269

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Diagnostic accuracy of rapid diagnostic tests for the early detection of leptospirosis

Siti N. Alia [a], Narcisse Joseph [a], Noraini Philip [a], Nurul N. Azhari [a], Bashiru Garba [a,b], Siti N. Masri [a], Zamberi Sekawi [a], Vasantha K. Neela [a,*]

[a] Department of Medical Microbiology and Parasitology, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[b] Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto, 840245 Nigeria

ARTICLE INFO

Article history:
Received 13 May 2018
Received in revised form 16 October 2018
Accepted 30 October 2018

Keywords:
Leptospirosis
Rapid diagnostic test
ImmuneMed Leptospira IgM Duo Rapid test kit
Leptocheck-WB rapid diagnostic kit
Malaysia

ABSTRACT

Background: Leptospirosis is often misdiagnosed with several other tropical febrile illnesses in Malaysia due to similarities in clinical manifestations. Although treatment regimens could be started based on clinical judgments, early diagnosis has become paramount as a guide to chemotherapeutic interventions. Confirmed laboratory diagnosis through MAT or PCR is time consuming and usually available only in reference laboratories and not practical in healthcare settings. Rapid and easy to perform diagnostic tests are widely used in these settings as the point of care diagnosis. The present study was undertaken to compare the diagnostic performance of two IgM based immunodiagnostic assay kits for acute leptospirosis.

Methods: A total of 50 serum samples were collected from patients clinically suspected for acute leptospirosis on admission in the Hospital Serdang, from June 2016 to June 2017. All the samples were subjected to MAT, *lipL32* PCR and the two rapid tests (Leptocheck-WB and ImmuneMed Leptospira IgM Duo Rapid test).

Results: Out of the 50 clinically suspected patients sampled, 19 were confirmed positive for leptospirosis. Six (12%) were confirmed by MAT and 13 (26%) by PCR. Similarly, of the 50 clinically suspected cases, 17 (34%) showed positivity for Leptocheck-WB and 7 (14%) for ImmuneMed Leptospira IgM Duo Rapid test. The overall sensitivity and specificity was 47.37% and 80.65% for Leptocheck-WB, and 21.05% and 90.32% for ImmuneMed Leptospira IgM Duo Rapid test. In another set of previously confirmed MAT positive samples (1:400–1:3600) obtained from a reference laboratory, Leptocheck-WB showed higher sensitivity (90.72%) than ImmuneMed Leptospira IgM Duo Rapid test (40.21%), and comparable specificity for ImmuneMed Leptospira IgM Duo Rapid test (88.89%) and Leptocheck-WB (82.86%).

Conclusion: The sensitivity was higher for Leptocheck-WB and had a comparable specificity with ImmuneMed Leptospira IgM Duo Rapid test. Therefore, based on the present study, Leptocheck-WB is found to be a more sensitive rapid immunodiagnostic test for acute leptospirosis screening in hospital settings.

Introduction

The neglected tropical illness leptospirosis caused by the spirochete *Leptospira*, is now an alarming re-emerging zoonosis with a worldwide distribution [1,2]. In Malaysia, leptospirosis is gazetted as a notifiable disease since December 2010 [3]. The number of cases according to the data from the Ministry of Health Malaysia (MOH) showed an increase from 3665 in 2012 to 5284 in 2016 [3,4]. Leptospirosis is a biphasic infection, the first phase (acute or septic phase) commences from 3 to 10 days of disease onset and the second phase (immune phase) ranges from 7 to 14 days [5]. During the acute phase, the bacteria can be found in the blood and then migrate and reside in the kidney where it continues to be shed in the urine. While in the second phase, a detectable number of antibodies develops and this stage coincides with the disappearance of the bacteria in the blood [6]. Confirmatory laboratory diagnosis for leptospirosis involves testing for antigen (bacteria by culture or PCR of *Leptospira* pathogenic genes) in the first phase and antibodies in the second phase through the gold standard microscopic agglutination test (MAT).

Culture is not suitable for early diagnosis as *Leptospira* takes two weeks to four months to grow [7]. On the other hand, PCR based

* Corresponding author.
E-mail address: vasantha@upm.edu.my (V.K. Neela).

https://doi.org/10.1016/j.jiph.2018.10.137

detection, although gives a confirmatory diagnosis, it involves DNA extraction, technical expertise, expensive PCR machines and reagents, which limits the feasibility in many health care facilities [8]. MAT is technically tedious and interpretations are very subjective and most importantly requires a greater panel of live leptospiral cultures to serve as antigens with the regular incorporation of new local and international serovars [9,10].

In Malaysia, MAT is available only at the National Leptospirosis Reference Centres such as the Institute for Medical Research located at capital Kuala Lumpur as well as the Zonal Public Health Laboratories. Considering all these shortcomings and challenges, a rapid test is highly desired and mostly preferred in hospitals in Malaysia, as it is a point of care test that can be performed in-house, and it is fast, technically simple and can be easily interpreted. It is pertinent to note that, there are several rapid tests commercially available which detect IgM antibodies produced against *Leptospira* antigens in the human serum. However, the sensitivity and the specificity vary for different kits in different geographical regions [9]. To date, only two studies have evaluated commercially available rapid diagnostic kits for acute leptospirosis in Malaysia [11,12]. One of these studies, evaluated two commonly used rapid leptospirosis serological tests in Malaysia, Leptorapide® (Linnodee, Northern Ireland) and VISITECT® -LEPTO (Omega Diagnostics, Scotland, UK) reported limited diagnostic value in detecting acute leptospirosis as they showed lower sensitivities and specificities [11]. However, a more recent evaluation study on the IgM Duo Rapid test kit from Korea (immunochromatographic assay), showed a diagnostic sensitivity of 73% and specificity of 90% [12]. Nonetheless, a point of note regarding the aforementioned studies is that, both of them were performed on previously confirmed MAT/PCR positive samples rather than a prospective clinical evaluation of samples from patients in a hospital setting. As elsewhere, in Malaysia few serovars isolated locally are frequently observed among patients. Hence, it is also important to determine the diagnostic efficacy of any RDT against the locally isolated serovars as well.

Therefore, it is crucial to identify a test that suits the particular setting. The present study aimed at evaluating and comparing the diagnostic accuracy of two rapid diagnostic tests (RDTs) Leptocheck-WB (Zephyr Biomedicals India) and ImmuneMed Leptospira IgM Duo Rapid test (ImmuneMed Inc., Republic of Korea) for the detection of *Leptospira* IgM antibodies through a prospective hospital study as a guide for health sectors to incorporate the most appropriate test in their routine diagnostic tools panel.

Materials and methods

Leptospirosis case classification

A clinical case of leptospirosis is defined as a patient who has a history of exposure to contaminated environment (in a leptospirosis endemic area), and presenting acute febrile illness accompanied by headache, myalgia, conjunctival suffusion, jaundice, hemorrhages (from the intestines and lungs) and gastrointestinal symptoms (Ministry of Health, Malaysia guidelines). Similarly, a patient is considered a probable case when he/she fulfills the above clinical case definition and is sero-positive based on ELISA or rapid test. while, a patient is thought of having confirmed leptospirosis, when a probable case is positive for any one of the following laboratory test;

1. MAT: A single serum titer ≥1:400 or paired serum with four-fold or greater rise (seroconversion).
2. Positive PCR, where the sample is collected within 10 days of disease onset.
3. Positive culture for pathogenic *leptospires* from blood samples taken within seven days of disease onset or from urine sample after the 10th day.
4. Demonstration of *Leptospira* antigen in tissues by immunohistochemical staining.

Inclusion and exclusion criteria

Inclusion criteria. Although history of contaminated environment exposure was asked, all patients with acute febrile illness were included in the study.

Exclusion criteria. Patients that showed clear symptoms or confirmed for other illness were excluded.

Patient and samples

The study was approved by the Medical Research and Medical Committee, Ministry of Health Malaysia (NMRR-15-2148-27536). A written informed consent was obtained from all patients participated in the study. Blood samples were collected from patients clinically suspected (as per MOH guidelines) for leptospirosis admitted at the Hospital Serdang from June 2016 to June 2017. All samples were collected from the acute phase. Blood samples were collected in a plain tube (serum for serology) and EDTA tubes (for PCR). The hospital is a multi-specialty 620 bedded tertiary healthcare located in the Sepang district in the state of Selangor, Malaysia. All samples were subjected to PCR targeting *lipL32* gene and MAT. All tests were performed on the blood samples taken on admission or within four days of admission. As a routine, after four days, most of the patients were discharged if no major complications. For few patients paired serum samples were available at three weeks to one-month interval. No cultures were available, hence is not included in the present study. In addition to the prospective samples from Hospital Serdang, 97 MAT confirmed leptospirosis non duplicate or non- paired serum samples were obtained from Public Health Laboratory of the Kelantan State Health Department (Covering North East Malaysia). All samples were obtained from acute leptospirosis, collected at the time of admission or when the patient is suspected for leptospirosis (personal communication with the Public Health Laboratory of the Kelantan State Health Department).

Laboratory methods

Polymerase chain reaction

DNA was extracted from the blood collected in EDTA tubes using the DNAeasy blood and tissue kit (Qiagen, Valencia, California, USA). All DNA samples (standardized at 10–20 ng/μl) were subjected to qPCR (QuantiNova Probe PCR, Qiagen, Dusseldorf, Germany) targeting the 242 bp *lipL32* gene fragment (LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and (LipL32-286R 5′-GAA CTC CCA TTT CAG CGA TT-3′)) [13].

Microscopic agglutination test

The MAT was performed for all samples with a panel of 20 serovars comprising of pathogenic and non-pathogenic leptospires. Local serovars were obtained from IMR (IMR LEP 1; saprophyte, IMR LEP 115; saprophyte, IMR LEP 175; saprophyte, IMR LEP 803/11-Copenhageni, IMR LEP 27-Hardjobovis, IMR LEP 22-Lai) and international panel (n = 14) from WHO Leptospirosis collaborating centre, Amsterdam (Australis, Autumnalis, Batavia, Canicola, Celledoni, Grippotyphosa, Hardjoprajitno, Icterohaemorrhagiae, Javanica, Pyrogenes, Tarrasovi, Djasiman, Patoc and Pomona).

Serum obtained from patients was diluted to 1:25 with phosphate buffered solution (PBS). Fifty microliter of the diluted serum was used to screen for agglutination against each serovar before

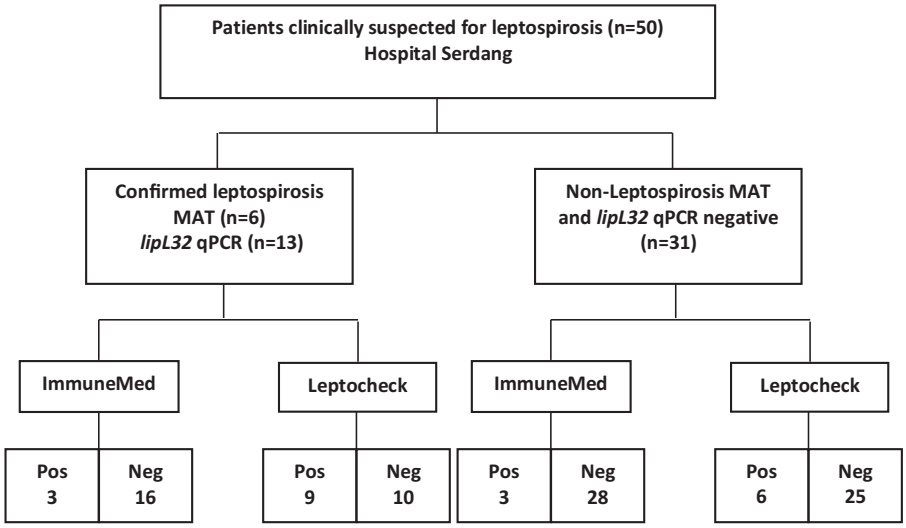


Fig. 1. Flowchart of participants and rapid diagnostic tests for prospective samples (Hospital Serdang samples).

performing the full MAT evaluation. Those serovars that gave positive agglutination were subjected to titration to determine the titer. Briefly, live *Leptospira* serovar cell suspensions (50 μl of 3+) in liquid EMJH (Ellinghausen- McCullough-Jonson-Haris) media were added to serially diluted (2 fold dilution for eg: well 1; 50 μl phosphate buffered solution as control, well 2; 50 μl of serum, well 3–6; 50 μl of 2 fold serially diluted serum with PBS) serum samples in a 96 well microtiter plate and incubated at 30 °C for 2 h. Agglutination was examined at 20× magnification under a dark field microscope (OLYMPUS BX53). Positive agglutination was considered when at least 50% of the *leptospires* agglutinate with the serum antibodies. The titer was recorded as the last dilution that showed <50% free *leptospires* compared to the control wells. True positive is defined as a sample which gave MAT titer of ≥1:400 for a single serum specimen or 4 fold seroconversion for paired samples (for eg: 50–200 or 100–400).

Rapid diagnostic test evaluation

Two immunochromatography based rapid tests; the Leptocheck-WB (Zephyr Biomedicals India) and ImmuneMed Leptospira IgM Duo Rapid test (ImmuneMed Inc., Republic of Korea) for diagnosis of leptospirosis were evaluated for their diagnostic accuracy in detecting cases of acute leptospirosis.

Leptocheck-WB. Leptocheck-WB is a rapid test kit for the detection of *Leptospira*-specific IgM antibodies in human serum/plasma/whole blood. The principle is based on the immunochromatographic agglutination of circulating antibodies in serum of patients with specific antigen using nano-gold particles as agglutination revealing agent. The test was performed according to the manufacturer's instruction. Briefly, 10 μl of serum was dispensed into the sample port A, followed by dropping 5 drops of running buffer (provided in the plastic dropper bottle) in the buffer port B. The results were read visually after 15 min of incubation at room temperature. The presence of red to purple coloured band in the test region 'T' and the control 'C' indicates positive results.

ImmuneMed Leptospira IgM Duo Rapid test. This is an immunochromatographic assay for semi-quantitative detection of IgM antibodies against Leptospira in the patient's serum/plasma/whole blood. This kit detects IgM antibody at two titers (1:50 for inconclusive and 1:200 for conclusive). Based on the manufacturer's protocol, 3 μl of serum was diluted to 100 fold with 300 μl of sample diluent. The diluted samples were then applied to the hole in the sample pad. Alternatively, 3 or 6 μl of the samples could be placed on the sample pad followed by the addition of 7 drops of the sample diluent. The result was observed after 15 min incubation at room temperature. The test was declared positive when a red line appears at the control line "C" and test lines "T" at 50 and 200, while only at "C" and T50 indicates inconclusive or intermediate.

Data analysis

Diagnostic accuracy was defined by sensitivity and specificity, false positivity and false negativity, true positivity and true negativity for each test. The diagnostic accuracy was determined using the following formulae:

Sensitivity (%) = True positives/(true positives + false negatives) × 100%

Specificity (%) = True negatives/(false positives + true negatives) × 100%

Positive predictive value (%) = True positive/(True positive + False positive) × 100%

Negative predictive value (%) = True negative/(False negative + True negative) × 100%

True positive: samples that showed positive for the confirmed (PCR/MAT) leptospirosis cases

True negative: samples that showed negative for the confirmed (PCR/MAT) non-leptospirosis cases

For the ImmuneMed Leptospira IgM Duo Rapid test kit, which gives conclusive and inconclusive results, only the conclusive result was interpreted as positive for calculating specificity and sensitivity. Proportions and 95% confidence interval were calculated with Medcalc software (https://www.medcalc.org). All inconclusive samples were repeated 2–5 days after the first sample to avoid any false negative results.

Results

In order to determine the efficacy of Leptocheck-WB and ImmuneMed Leptospira IgM Duo Rapid test, the blood sample collected from each participating patient was validated in the Microbiology laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. A sample which is either PCR or MAT (titer > 1:400 or seroconverted) or both positive is considered as confirmed leptospirosis. While a sample which is negative by MAT/PCR is considered non-leptospirosis illness. In total, 19 out

Table 1
Comparison of RDTs with reference tests.

Patient	Admission sample				Discharge sample			
	qPCR	MAT	LC	IM	qPCR	MAT	LC	IM
1	–	–	+	+	NA	+	+	+
2	–	–	–	–	NA	–	–	–
3	+	–	–	–	NA	–	–	–
4	–	+	+	–	NA	NA	NA	NA
5	–	–	–	–	NA	NA	NA	NA
6	–	–	–	–	NA	NA	NA	NA
7	–	+	–	–	NA	NA	NA	NA
8	–	+	–	–	NA	NA	NA	NA
9	–	–	+	–	NA	–	+	–
10	–	+	–	–	NA	+	–	–
11	–	–	–	–	NA	–	–	–
12	–	+	+	+	NA	NA	NA	NA
13	–	–	–	–	NA	–	–	–
14	–	–	+	–	NA	NA	NA	NA
15	–	–	+	+	NA	–	+	+
16	–	–	+	+	NA	–	+	+
17	–	–	–	–	NA	–	–	–
18	–	–	–	–	NA	NA	NA	NA
19	–	–	+	+	NA	NA	NA	NA
20	–	–	+	+	NA	–	–	–
21	–	–	–	–	NA	NA	NA	NA
22	+	–	–	–	NA	NA	NA	NA
23	+	–	–	–	NA	–	–	–
24	–	–	–	–	NA	NA	NA	NA
25	–	–	–	–	NA	–	–	–
26	–	–	–	–	NA	NA	NA	NA
27	+	–	–	–	NA	–	+	–
28	+	–	–	–	NA	NA	NA	NA
29	+	-	+	+	NA	NA	NA	NA
30	+	–	–	–	NA	–	+	–
31	+	–	–	–	NA	–	+	–
32	+	–	–	–	NA	NA	NA	NA
33	+	–	+	–	NA	–	+	–
34	–	–	–	–	NA	NA	NA	NA
35	–	–	–	–	NA	NA	NA	NA
36	–	–	+	–	NA	–	–	–
37	–	–	–	–	NA	–	–	–
38	–	–	+	–	NA	NA	NA	NA
39	–	–	–	–	NA	–	–	–
40	–	–	–	–	NA	NA	NA	NA
41	–	–	–	–	NA	–	–	–
42	–	–	–	–	NA	–	+	–
43	–	–	+	–	NA	–	+	+
44	+	–	–	–	NA	–	–	–
45	+	–	–	–	NA	–	–	–
46	–	–	–	–	NA	–	–	–
47	+	–	+	–	NA	NA	NA	NA
48	–	–	–	–	NA	NA	NA	NA
49	–	–	+	–	NA	–	+	–
50	–	–	–	–	NA	–	–	–

of the 50 clinically suspected patients were confirmed positive for leptospirosis. Six (12%) were confirmed by MAT (five positive on the day of admission and one on the day of discharge which was 2 days post admission) and 13 (26%) by PCR (Fig. 1). For rapid test all patients who showed negative on day one were repeated with discharge sample taken within 2–5 days of the first sample (Table 1).

Although among the 50 clinically suspected cases, 17 (34%) showed positivity for Leptocheck-WB and 7 (14%) for ImmuneMed Leptospira IgM Duo Rapid test, only 9 (18%) (Six by admission sample and three by discharge sample) were true positive for Leptocheck-WB and 3 (6%) for ImmuneMed Leptospira IgM Duo Rapid test (three by admission sample and one by discharge sample). For ImmuneMed Leptospira IgM Duo Rapid test, only conclusive was interpreted as positive (Table 2). For all inconclusive samples, except for one sample, the result remained the same when repeated on the discharge samples.

Overall sensitivity and specificity was 47.37% and 80.65% for Leptocheck-WB and 21.05% and 90.32% for ImmuneMed Leptospira IgM Duo Rapid test. Of the 9 Leptocheck-WB positive patients, three were positive by MAT and six by PCR and none showed positivity by all three methods. Of the four ImmuneMed Leptospira IgM Duo Rapid test conclusive samples, two were positive for MAT, one for PCR and none by all methods.

Table 2
Comparison of results of immunochromatographic (Leptocheck-WB and ImmuneMed Leptospira IgM Duo Rapid test), MAT and PCR tests for patient samples from Hospital Serdang.

Test	MAT (n = 6)	PCR (n = 13)	Sensitivity	Specificity
Leptocheck-WB (n = 9)	3	6	47.37%	80.65%
ImmuneMed Leptospira IgM Duo Rapid test (n = 3)	2	1	15.79%	90.32%

Table 3
Comparison of results of immunochromatographic (Leptocheck and ImmuneMed Leptospira IgM Duo Rapid test), MAT and PCR tests for leptospirosis confirmed samples from Public Health laboratory.

Test	MAT (n = 97)	Sensitivity	Specificity
Leptocheck-WB	88	90.72%	76.32%
ImmuneMed Leptospira IgM Duo Rapid test	39	40.21%	89.47%

Table 4
Overall spensitivity and specificity of the rapid tests.

Test	Study type	Sensitivity	CI	Specificity	CI
Leptocheck-WB	Prospective	47.37%	24–71	80.65%	62–92
ImmuneMed	Prospective	15.79%	3–39	90.32%	74–97
Leptocheck-WB	Retrospective	90.72%	83–95	76.32%	59–88
ImmuneMed	Retrospective	40.21%	30–50	89.47%	75–97

In addition to the above samples, the kits were also evaluated against a panel of MAT confirmed positive serum (titers from 1:400 to 1:3600) obtained from a public health laboratory (all samples were collected during admission or when patients were suspected for leptospirosis) and MAT negative serum (from healthy individuals and other febrile illnesses) (Fig. 2). Among the two tests, Leptocheck-WB showed a higher sensitivity of 90.72% compared to ImmuneMed Leptospira IgM Duo Rapid test (40.21%) (Table 3). On the other hand, the specificity was higher for ImmuneMed Leptospira IgM Duo Rapid test (89.47%) compared to Leptocheck-WB (76.32%) (Table 3). Among the five dengue positive samples tested, one showed a positive signal for Leptocheck-WB. Four out of twenty-five (4/25) healthy controls (MAT negative) were found to be positive by Leptocheck-WB while only two were conclusive by ImmuneMed Leptospira IgM Duo Rapid test respectively. Leptocheck-WB detected comparatively more number 5/10 (50%) of local serovar IMR LEP 175 compared to 3/10 (30%) by ImmuneMed Leptospira IgM Duo Rapid. Another finding to be noted include Leptocheck-WB detected more number of samples for all titers (49/53 MAT 400; 28/32 MAT 800, 8/9 MAT 1600; 3/3 for MAT 3200) when compared to ImmuneMed Leptospira IgM Duo Rapid test (17/53 for MAT400; 15/32 for MAT 800; 5/9 for MAT 1600 and 2/3 for MAT 3200) (Table 4).

Discussion

The urgent need for the development of rapid diagnostic assays has led to the proliferation of a number of rapid kits with varying level of sensitivity and specificity. The choice for suitable test kit depends on the regional prevalence of disease, cost, and availability of the kits. Despite the many claims of effectiveness, many of

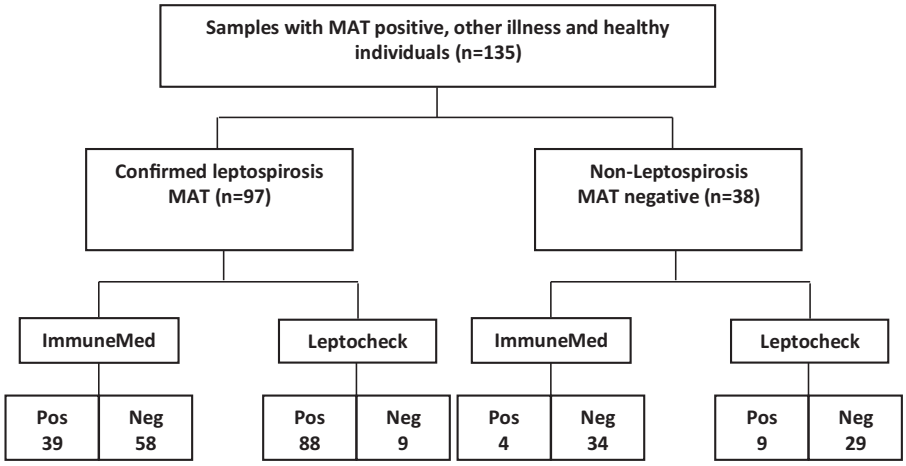


Fig. 2. Flow chart of participants and rapid diagnostic tests for retrospective samples (Public Health Laboratory Kota Bharu and Healthy individual samples).

these kits upon comparative evaluation have produced inconsistent results [7,14]. Despite Malaysia being a tropical country with increasing cases of human leptospirosis, there are not many reports on the most sensitive and specific rapid test for early screening of the illness. A study conducted in 2014 by Chang and colleagues [11] concluded the two (Leptorapide® from Linnodee, Northern Ireland and VISITECT®-LEPTO kit manufactured by Omega Diagnostics Group PLC, Scotland, UK) commonly used commercial rapid tests for acute leptospirosis in Malaysia have limited diagnostic value. A recent study by Amran et al. [12] showed a diagnostic sensitivity of 73% and specificity of 90% for IgM Duo Rapid test kit from Korea on retrospective Leptospirosis confirmed samples. The two important challenges that proper evaluation of a diagnostic test has to address are; well-defined status of the samples to be subjected for validation particularly with regards to the diagnostic target and the results produced must compare favorably with results of other reference tests conducted with the same set of samples; in this case, the gold standard MAT for leptospirosis and PCR. Hence, the present study was aimed at evaluating Leptocheck-WB and ImmuneMed Leptospira IgM Duo Rapid test for its usefulness in acute leptospirosis screening in the hospital. Traditionally, the characteristics features of good rapid diagnostic tests should include; accuracy, ease of use without any technical skill or equipment, affordability and easy to interpret results. Other essential features, especially for application in tropical regions where the disease is endemic, are; temperature stability and produce results within a short period of time [8]. Both RDT's evaluated were easy to perform, do not need any technical expertise or special equipment or conditions, and could be done bedside and results obtained as early as 15 min. This type of convenient testing is very important for early screening in hospitals or clinics.

To determine the usefulness of Leptocheck-WB or ImmuneMed Leptospira IgM Duo Rapid test as a screening test for the detection of leptospirosis, their performance on diagnostic serum samples obtained from patients clinically suspected for leptospirosis was evaluated. Although 50 patients clinically suspected, only 19 were confirmed for leptospirosis based on laboratory diagnoses such as MAT (n = 6) and PCR (n = 13). Since paired sera obtained between 2 and 30 days after the first sample was available for only 22 samples, the actual number of MAT positive sera in the present study may not be accurate. In addition, sharing of similar symptoms or coinfection with endemic dengue, the number of clinically suspected leptospirosis cases could be more than true leptospirosis cases. However, when considering the sensitivity, Leptocheck-WB (47.37%) was superior to ImmuneMed Leptospira IgM Duo Rapid test (15.79%). On the other hand, ImmuneMed Leptospira IgM Duo Rapid test (90.32%) was found to be more specific than Leptocheck-WB (80.65%). One of the contributing factor for vast low sensitivity could be because the majority of samples were positive by PCR, which is antigen-based detection that occurs in the spiremia phase rather than immune phase eliciting antibody production. We observed a high positivity for PCR; this could be because as soon the patient is clinically suspected, the blood samples were collected and processed within 2 h for DNA extraction and performed PCR. Prior screening of various published PCR targets and protocols revealed the Taqman PCR protocol utilized herein as the most sensitive assay. None of the PCR detected were MAT positive as antibody rise is usually observed after the organism is eliminated from the blood (immune phase) [15]. In most cases, the antibiotic therapy is started when a patient is clinically suspected of leptospirosis, which may also interrupt the synthesis of antibodies resulting in inconclusive serological results [16].

An additional set of samples that were previously confirmed by MAT, (titers between 1:400 to 3600) were also evaluated with the kits. These samples were obtained from patients in the north-eastern states of Peninsular Malaysia where leptospirosis is highly endemic [3,4]. MAT titers of 1:400 for a single sample is set for confirmed leptospirosis in Malaysia according to the guidelines of the Ministry of Health, Malaysia where leptospirosis is endemic [17–19]. The higher cut off MAT antibody titer as diagnostic is usually set in an endemic area like Malaysia especially where the potential risk factors abound [11].

Among the two RDT's evaluated, Leptocheck-WB (90.27%) showed the highest sensitivity compared to ImmuneMed Leptospira IgM Duo Rapid test (40.21%). A good sensitivity was observed for samples with MAT titers of more than 800 by both RDT's. The nine MAT positive samples that showed negative for Leptocheck-WB included one Patoc, five IMR LEP 175 (local serovar yet to be officially named) and one each of batavia and icterohaemorrhagiae. On the other hand, the vast majority of ImmuneMed Leptospira IgM Duo Rapid test negative comprised celledoni, batavia, grippotyphosa, icterohaemorrhagiae, and IMR LEP 175 serovars. Majority of the samples which showed negative by both test had MAT titers of 1:400. Despite being MAT positive, the two tests were not able to detect antibodies against the aforementioned serovars. While the fact that, IMR LEP 175 is a saprophytic pathogen and their lack of activity in the blood and absence of antigenic markers synonymous with lower detection, Leptocheck-WB detected more (5/10; 50%) such strains (local serovars) compared to ImmuneMed Leptospira IgM Duo Rapid test (3/10; 30%). In addition, the conclusion to judge single test titers of 1:400 as positive is questionable and counters majority judgement where a four-fold

rise in antibody titer upon the second test due to seroconversion is considered definitive, especially considering the low sensitivity of MAT [20,21]. For ImmuneMed Leptospira IgM Duo Rapid test, only 39 samples were detected as conclusive for MAT titers that ranged from 400 to 3600. Leptocheck-WB identified 26 (83.9%) of the 31 MAT negative sera (healthy controls and other tropical illness) as negative, while ImmuneMed Leptospira IgM Duo Rapid test detected 30 (96.8%) as negative. Therefore, the evaluation with both set of samples, it is convincing that Leptocheck-WB is more suitable for screening of acute leptospirosis in Malaysia. The fact that Leptocheck-WB is developed based on the broadly reactive genus-specific antigen, may be responsible for the superior sensitivity, permitting the kit to detect *Leptospira* infections caused by a wide range of strains belonging to different serovars as against ImmuneMed Leptospira IgM Duo Rapid test tested in this study.

The usefulness of Leptocheck-WB in screening for acute leptospirosis have been reported in several studies from Slovenia [22], India [23,24] and Sri Lanka [25,26]. In contrast, only two studies have been reported on ImmuneMed Leptospira IgM Duo Rapid test [12,27]. The clinical evaluation of ImmuneMed Leptospira IgM Duo Rapid test in Korea, Bulgaria, and Argentina showed the sensitivity of 93.9%, 100% and 81% and specificity of 97.9%, 100% and 95.4% [27]. However, this is the earlier version of the kit, where the IgM at MAT 1:100 were set as positive compared to the 1:200 (conclusive in the ImmuneMed Leptospira IgM Duo Rapid test kit available in Malaysia).

In conclusion, of the two RDT's evaluated, Leptocheck-WB was found to be more sensitive than ImmuneMed Leptospira IgM Duo Rapid test. Results on ImmuneMed Leptospira IgM Duo Rapid test inconclusive is also important herein mainly to focus on these patients for a second sample a few days later or on the convalescent serum to avoid false negative results. Prospective evaluation of clinically suspected cases gives actual sensitivity and specificity than retrospective confirmed sample evaluation. However, evaluation with larger sample size covering all regions of Malaysia would give a clearer picture of the most suitable rapid test. The ease of performance without specialized equipment and affordable cost supports its usefulness and preference in hospitals and the laboratories for the screening of acute leptospirosis. However, the failure to detect PCR positive samples strongly recommends the development of new RDTs with antigen and antibody detection in the same test.

Funding

This work was supported by the Long Term Research Grant Scheme (LRGS Phase 2/2014, UPM/700-2/7/LRGS/5526400).

Competing interests

None declared.

Ethical approval

Required.

Acknowledgements

We take this opportunity to thank the Ministry of Higher Education and Universiti Putra Malaysia through Long Term Research Grant Scheme (LRGS Phase 2/2014, UPM/700-2/7/LRGS/5526400). We also wish to acknowledge the Kelantan State Health Department, Hospital Serdang, Institute for Medical Research and the Ministry of Health Malaysia, for providing samples, and their contribution and support throughout the project. Authors thank members of GLEAN (Global Leptospirosis Environmental Action Network) and WHO reference centre, Amsterdam, The Netherlands for guidance throughout the study. We would like to thank the Director General of Health, Malaysia for his permission to publish this data.

References

[1] Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol 2015;387:65–97, http://dx.doi.org/10.1007/978-3-662-45059-8_5.
[2] Levett PN. Leptospirosis. Clin Microbiol Rev 2001:14, http://dx.doi.org/10.1128/CMR.14.2.296.
[3] Benacer D, Thong KL, Verasahib Bin K, Galloway RL, Hartskeerl RA, Lewis JW, et al. Human Leptospirosis in Malaysia. Asia Pacific J Public Health 2016;28:290–302, http://dx.doi.org/10.1177/1010539516640350.
[4] Garba B, Bahaman AR, Khairani-Bejo S, Zakaria Z, Mutalib AR. Retrospective study of Leptospirosis in Malaysia. Ecohealth 2017;14:389–98, http://dx.doi.org/10.1007/s10393-017-1234-0.
[5] Picardeau M. Diagnosis and epidemiology of leptospirosis. Médecine Mal Infect 2013;43:1–9, http://dx.doi.org/10.1016/j.medmal.2012.11.005.
[6] Tereza Cristina MM, Silva TCC. Pathogenesis of Leptospirosis: important issues. J Med Microbiol Diagn 2015;04, http://dx.doi.org/10.4172/2161-0703.1000e127.
[7] Levett PN. Systematics of leptospiraceae. Curr Top Microbiol Immunol 2015;387:11–20, http://dx.doi.org/10.1007/978-3-662-45059-8_2.
[8] Musso D, La Scola B. Laboratory diagnosis of leptospirosis: a challenge. J Microbiol Immunol Infect 2013;46:245–52, http://dx.doi.org/10.1016/j.jmii.2013.03.001.
[9] Picardeau M, Bertherat E, Jancloes M, Skouloudis AN, Durski K, Hartskeerl RA. Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies. Diagn Microbiol Infect Dis 2014;78:1–8, http://dx.doi.org/10.1016/j.diagmicrobio.2013.09.012.
[10] Hartskeerl RA, Smythe LD. The role of leptospirosis reference laboratories. Curr Top Microbiol Immunol 2015;387:273–88, http://dx.doi.org/10.1007/978-3-662-45059-8_11.
[11] Chang CH, Amran F, Riazi M, Yunus MH, Osman S, Noordin R. Evaluation of two commercial rapid tests for laboratory diagnosis of Leptospirosis in Malaysia. Asian Pacific J Trop Dis 2014;4:246, http://dx.doi.org/10.1016/S2222-1808(14)60557-0.
[12] Amran F, Liow YL, Halim NAN. Evaluation of a commercial immunochromatographic assay kit for rapid detection of igm antibodies against Leptospira antigen in human serum. J Korean Med Sci 2018;33:e131, http://dx.doi.org/10.3346/jkms.2018.33.e131.
[13] Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR. Detection of pathogenic Leptospira spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. Diagn Microbiol Infect Dis 2009;64:247–55, http://dx.doi.org/10.1016/j.diagmicrobio.2009.03.014.
[14] 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, http://dx.doi.org/10.1093/cid/cis403.
[15] Goris MGA, Kikken V, Straetemans M, Alba S, Goeijenbier M, van Gorp ECM, et al. Towards the burden of human leptospirosis: duration of acute illness and occurrence of post-leptospirosis symptoms of patients in the Netherlands. PLoS One 2013;8:e76549, http://dx.doi.org/10.1371/journal.pone.0076549.
[16] Terpstra WJ, World Health Organization. Human leptospirosis: guidance for diagnosis, surveillance and control. Geneva, Switzerland: World Health Organization; 2003, http://www.who.int/iris/handle/10665/42667.
[17] El Jalii IM, Bahaman AR. A review of human leptospirosis in Malaysia. Trop Biomed 2004;21:113–9.
[18] Lim JK, Murugaiyah VA, Ramli AS, Rahman HA, Mohamed NSF, Shamsudin NN, Tan JC. A Case Study: Leptospirosis In Malaysia. Infect Dis 2011;2(12). WMC00276.
[19] Thayaparan S, Robertson ID, Fairuz A, Suut L, Abdullah MT. Leptospirosis, an emerging zoonotic disease in Malaysia. Malaysian J Pathol 2013;35:123–32.
[20] Chirathaworn C, Inwattana R, Poovorawan Y, Suwancharoen D. Interpretation of microscopic agglutination test for leptospirosis diagnosis and seroprevalence. Asian Pac J Trop Biomed 2014;4:S162–4, http://dx.doi.org/10.12980/APJTB.4.2014C580.
[21] Brandão AP, Camargo ED, da Silva ED, Silva MV, Abrão RV. Macroscopic agglutination test for rapid diagnosis of human leptospirosis. J Clin Microbiol 1998;36:3138–42.
[22] Podgoršek D, Cerar T, Logar M, Lešničar G, Remec T, Baklan Z, et al. Evaluation of the immunochromatographic (Leptocheck) test for detection of specific antibodies against leptospires. Wien Klin Wochenschr 2015;127:948–53, http://dx.doi.org/10.1007/s00508-015-0853-0.
[23] Panwala T, Rajdev S, Mulla S. To evaluate the different rapid screening tests for diagnosis of leptospirosis. J Clin Diagn Res 2015;9, http://dx.doi.org/10.7860/JCDR/2015/11188.5587. DC21-4.
[24] Gajjar A, Sarwankar, Mulla S. Role of rapid serological tests in the diagnosis of acute leptospirosis in south gujarat region, India. Int J Adv Sci Eng Technol 2017;19:978–93.

[25] Niloofa R, Fernando N, de Silva NL, 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, http://dx.doi.org/10.1371/journal.pone.0129236.

[26] Eugene EJ, Handunnetti SM, Wickramasinghe SA, Kalugalage TL, Rodrigo C, Wickremesinghe H, et al. Evaluation of two immunodiagnostic tests for early rapid diagnosis of leptospirosis in Sri Lanka: a preliminary study. BMC Infect Dis 2015;15:319, http://dx.doi.org/10.1186/s12879-015-1080-z.

[27] Lee J-W, Park S, Kim SH, Christova I, Jacob P, Vanasco NB, et al. Clinical evaluation of Rapid Diagnostic Test kit using the Polysaccharide as a genus-specific diagnostic antigen for Leptospirosis in Korea Bulgaria, and Argentina. J Korean Med Sci 2016;31:183–9, http://dx.doi.org/10.3346/jkms.2016.31.2.183.

Rapid diagnostic test (Leptocheck-WB) for detection of acute leptospirosis: a meta-analysis of diagnostic accuracy

Article *in* European Journal of Clinical Microbiology & Infectious Diseases · April 2022

DOI: 10.1007/s10096-022-04420-9

CITATION
1

READS
149

5 authors, including:

Htet Htet
International Medical University (IMU)
45 PUBLICATIONS **120** CITATIONS
SEE PROFILE

Cho Naing
119 PUBLICATIONS **1,742** CITATIONS
SEE PROFILE

Siew Tung Wong
International Medical University (IMU)
23 PUBLICATIONS **264** CITATIONS
SEE PROFILE

JW Mak
International Medical University (IMU)
366 PUBLICATIONS **4,001** CITATIONS
SEE PROFILE

European Journal of Clinical Microbiology & Infectious Diseases
https://doi.org/10.1007/s10096-022-04420-9

ORIGINAL ARTICLE

Rapid diagnostic test (Leptocheck-WB) for detection of acute leptospirosis: a meta-analysis of diagnostic accuracy

Siau Ek Hwee[1] · Norah Htet Htet[1] · Cho Naing[2] · Wong Siew Tung[1] · Joon Wah Mak[1]

Received: 23 November 2021 / Accepted: 4 February 2022

Abstract

The majority of leptospirosis is subclinical or mild self-limiting systemic illness. A rapid and accurate diagnostic test for the detection of leptospirosis is essential to prevent disease progression from acute non-severe illness to potentially fatal infection. Rapid diagnostic tests (RDTs) recognized as point-of-care (PoC) tests may support clinical decision-making in resource-poor settings. We aimed to assess the accuracy of PoC (Leptocheck-WB) for the detection of acute leptospirosis by meta-analysis of data from eligible studies. This study adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis for Diagnostic Test Accuracy (PRISMA-DTA) guideline. The pooling of data was done only when there were two or more studies that used a particular type of reference test. A total of ten studies ($n=5369$) were identified. The majority (70%) were from the Asian region. Using microscopic agglutination test (MAT) as reference test, the pooled sensitivity (0.75, 95% *CI*: 0.64 to 0.84, 10 studies, I^2: 85.9%) and specificity (0.87, 95% *CI*: 0.77 to 0.94, 10 studies, I^2: 97.37%) of Leptocheck-WB in the detection of leptospirosis were moderate. With the use of enzyme-linked immunosorbent assay (ELISA) reference test, the pooled sensitivity 0.85 (95% *CI*: 0.79 to 0.9, 4 studies, I^2: 27.49%) and specificity 0.79 (95% *CI*: 0.71 to 0.85, 4 studies, I^2: 58.9%) of Leptocheck-WB were also moderate. Diagnostic odds ratio of Leptocheck-WB with MAT (21, 95% *CI*: 10–44) or with ELISA as reference test (21, 95% *CI*: 9–46) showed an acceptable level of accuracy. Meta-regression analysis showed methodological quality of studies (p: 0.06) and study design (p: 0.09) were potential factors that affected the accuracy of Leptocheck-WB test. Findings suggest that Leptocheck-WB has a moderate level of acceptance for detection of acute leptospirosis. Further confirmation with large-sampled, prospectively designed studies using the same samples for evaluating test accuracy is recommended.

Keywords Leptospirosis · Test accuracy · Leptocheck-WB · Meta-analysis

Abbreviations

AJOL	African Journals online
AUC	Under the curve
DOR	Diagnostic odds ratio
ELISA	Enzyme-linked immunosorbent assay
FN	False negative
FP	False positive
GBD	Global burden of disease
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LILACs	Latin American and Caribbean Health Sciences Literature
MAT	Microscopic agglutination test
MeSH	Medical Subject Headings
PCR	Polymerase chain reaction
PoC	Point-of-care
PoCTs	Point-of-care tests
PRISMA-DTA	Preferred Reporting Items for Systematic Reviews and Meta-Analysis for Diagnostic Test Accuracy
QUADAS-2	Quality Assessment of Diagnostic Accuracy Studies-2
RDTs	Rapid diagnostic tests
ROC	Receiver operating characteristics
SROC	Summary receiver operating characteristics

Cho Naing was at the IMU during this study.

Norah Htet Htet and Wong Siew Tung contributed equally.

✉ Cho Naing
cho3699@gmail.com

[1] International Medial University, Kuala Lumpur, Malaysia

[2] Division of Tropical Health and Medicine, James Cook University, Queensland, Australia

Published online: 11 February 2022

TN	True negative
TP	True positive

Introduction

Leptospirosis is the most important zoonosis in the world, which is caused by spirochetes of the genus *Leptospira* [1]. *Leptospira* can spread through the urine of infected animals in water or soil. It has a broad geographical distribution [2] as rodent, cattle, pigs, horses, dogs and wild animals act as the reservoirs of the spirochete [1]. The highest estimates of leptospirosis morbidity and mortality listed in the Global Burden of Disease (GBD) are in the regions of South and Southeast Asia, Oceania, Caribbean, Andean, Central and Tropical Latin America and East Sub-Saharan Africa [2], leading to increased hospital admissions and a huge public health threat [3, 4]. A systematic review reported that 73% of the world's leptospirosis cases and deaths occur in countries situated between the Tropics of Cancer and Capricorn [2]. Furthermore, clinically leptospirosis may present as flu-like symptoms or misdiagnosed as dengue and malaria in endemic regions [2, 3]. A systematic review on GBD of morbidity and mortality showed that a considerable proportion of cases (48%) and deaths (42%) were in adults aged 20–49 years [2], reflecting a huge socio-economic loss of learning and earning capacities. Studies reported that the mortality is high in between 5 and 20% [4] or very high (> 50%) with severe pulmonary haemorrhage syndrome, albeit with optimal treatment [5]. Early confirmation and prompt treatment for acute leptospirosis are therefore crucially important.

There are various diagnostic approaches to confirm clinically suspected cases; direct detection methods include polymerase chain reaction (PCR) and isolation of *Leptospira* using culture as well as serological techniques such as the microscopic agglutination test (MAT) and immunoglobulin M enzyme-linked immunosorbent assay (IgM ELISA) to detect the presence of anti-*Leptospira* Immunoglobulin M (IgM and Immunoglobulin G (IgG)) [6, 7]. For confirmation, WHO recommends a fourfold or greater rise in titre or seroconversion in MAT on paired samples obtained at least 2 weeks apart and a positive PCR result using a validated method [8]. However, these methods have limitations especially in resource-limited regions. For instance, MAT is both laborious and complicated requiring live *Leptospira* [2, 7]. Furthermore, MAT shows cross-reactivity among several *Leptospira* [9]. IgM ELISA has low sensitivity in early infection. A systematic review on human leptospirosis incorporating 52 studies showed a pooled sensitivity of 0.86 (95% *CI*, 0.85–0.87) [10]. This implied a 14% chance of missing those who were true positives. Another systematic review of 42 studies for the detection of leptospirosis based on the *secY* gene had a low sensitivity of 0.56 (95% *CI*: 0.50–0.63) [11], indicating a 44% chance of missing true positives. As a better diagnostic method is not yet available and as *Leptospira* is a pathogen having more than 200 serovars, a simple, rapid and accurate diagnostic method is necessary [12].

The majority of leptospirosis is subclinical or mild self-limiting systemic illness [1, 3]. Hence, a rapid and accurate diagnostic test for the detection of leptospirosis is essential to prevent disease progression from acute non-severe illness to potentially fatal infection [10, 13]. The ideal diagnostic test, particularly in resource-poor settings should provide a rapid result with high sensitivity and specificity in the first few days of symptoms. As such, rapid diagnostic tests (RDTs) that are recognized as point-of-care (PoC) tests may be appropriate to support clinical decision-making in resource-poor settings [14]. Leptocheck-WB is an onsite, rapid and qualitative diagnostic kit and in brief, it utilizes the principle of agglutination of antibodies with the respective antigen in immunochromatography format with the use of nanogold particles to detect agglutination [15].

Overall, the objective of this study was to assess the accuracy of PoC Leptocheck-WB for the detection of acute leptospirosis by meta-analysis of data from eligible studies. The current study aimed to aid the process of early and accurate detection of acute leptospirosis in endemic areas. This will in turn contribute to improve medical service through early treatment and subsequent prevention from progression to severe acute leptospirosis and mortality.

Materials and methods

The present study adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis for Diagnostic Test Accuracy (PRISMA-DTA) guideline [16]. A completed PRISMA-DTA checklist is presented in Additional File 1. A protocol of this meta-analysis study was approved by the Institutional Joint Committee on Research and Ethics (ID: 353/2020).

Study search

Data source

The relevant studies were searched in the relevant electronic database such as PubMed, Ovid, Cochrane database, African Journals online (AJOL) and Latin American and Caribbean Health Sciences Literature (LILACs).

Search strategy

The search strategy was created using Medical Subject Headings (MeSH) terms with Boolean operators:

"Leptospirosis" OR "lepto", AND "immunochromatography test" OR, "Leptocheck-WB" AND "human". The search strategy used in PubMed was *((("leptospirosis"[MeSH Terms] OR "leptospirosis"[All Fields]) AND (("chromatography, affinity"[MeSH Terms] OR ("chromatography"[All Fields] AND "affinity"[All Fields]) OR "affinity chromatography" OR "immunochromatography" AND ("research design"[MeSH Terms] OR ("research" AND "design"[All Fields]) OR "research design" OR "test" OR Leptocheck-WB AND ("humans"[MeSH Terms] OR "humans" OR "human"*.

Search was limited to English until August 2021. We also performed a manual search in the references of the potentially eligible studies and relevant systematic reviews.

Study selection

Inclusion criteria for eligible studies were as described below.

Types of studies

Diagnostic studies that detected human acute leptospirosis, regardless of study design and geographic location.

Participants

Participants of the studies were suspected patients, regardless of age and gender.

Index test

Leptocheck-WB, regardless of manufacturers.

Reference tests

Currently available reference tests such as MAT, PCR or IgM-based microplate ELISA was regarded as reference standard. The reference standard was required to be done with the use of the same blood samples that were collected for the index test.

Target condition

Acute leptospirosis, as defined in the primary studies.

Outcome

The outcome of interest was diagnostic accuracy measured based on sensitivity, specificity and diagnostic odds ratio (DOR) of the index test.

Eligible studies therefore must have data on true positive (TP), true negative (TN), false positive (FP) and false negative (FN) to create a two-by-two table.

Sensitivity refers to the probability that the index test result is positive in infected cases. Specificity refers to the probability that index test result is negative in a non-infected case [17, 18].

Exclusion criteria

Studies were excluded if they did not meet the inclusion criteria. Therefore, studies without data to create a two-by-two table and diagnostic accuracy studies with animal models were not included. Studies on special groups of population like pregnant women were not considered. This is due to potential perturbation of immunity in pregnant women, making them physiologically different from the general population [19].

Data extraction

One investigator (SEH) screened title and abstracts, following the inclusion criteria set for this review. The same investigator extracted information from all included studies. Data collected were first author, publication year, country, setting, characteristic of study (sample size, details of tests employed, etc.), characteristic of study participants and outcome data (TP, FP, TN, FN). Information collected was cross-checked by another investigator (CN). In addition, the funding for studies and any conflicts of interest were noted. Throughout this data extraction process, any discrepancy between the two investigators was settled through discussion with the third investigator (NHH/WST).

QUADAS-2 risk of bias assessment

The methodological quality of included studies was assessed with the use of Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) checklist. This tool has four standard domains ("patient selection", "index tests", "reference standards" and "flow and timing"). Signalling questions under each domain were used and the answers for these signalling questions allowed the assessment of the risk of bias for each domain. The domains 1, 2 and 3 were also assessed for "applicability" [20, 21].

Data synthesis

The pooling of data was done only when there were two or more studies that used a particular type of reference test. As described elsewhere [18], sensitivity and specificity for each included study were described in the forest plots. A summary performance estimate was stratified by type of

reference tests. A bivariate model was used for data analyses. Summary receiver operating characteristic (SROC) plots were created to display the results of individual studies in a receiver operating characteristic (ROC) space. This indicates information on the overall performance of a test across different thresholds. The best diagnostic test is positioned in the top left-hand corner of the ROC space, whereby both the sensitivity and specificity are close to 1.0 [17, 22]. The area under the curve (AUC) represents the analytic summary of the diagnostic test performance among the included studies. An AUC of ≥ 0.97 indicates an excellent accuracy [17]. To detect source of heterogeneity, a meta-regression analysis was performed with covariates (i.e. sample size, study design, risk of bias and reference test type). A $p < 0.1$ in the joint model was considered to contribute to heterogeneity. Following PRISMA-DTA statement [16], the publication bias was not assessed by inspection of a funnel plot. All statistical analyses were done with midas package in STATA 15.0 (Statacorp, Txt) and RevMan 5.4 (The Nordic Cochrane Centre).

Results

Figure 1 shows the selection process of studies identified for the current meta-analysis. The initial search in the electronic databases yielded 811 citations. After removal of duplicates and further screening through title and abstract, a total of 23 articles were eligible for full-text evaluation. A final ten articles (17 datasets) were eligible for the current study [7, 15, 23–30]. All ten studies assessed with MAT, while only four studies were with ELISA [7, 25, 27, 28]. The reasons of exclusion of 13 studies were summarized in Additional file 2.

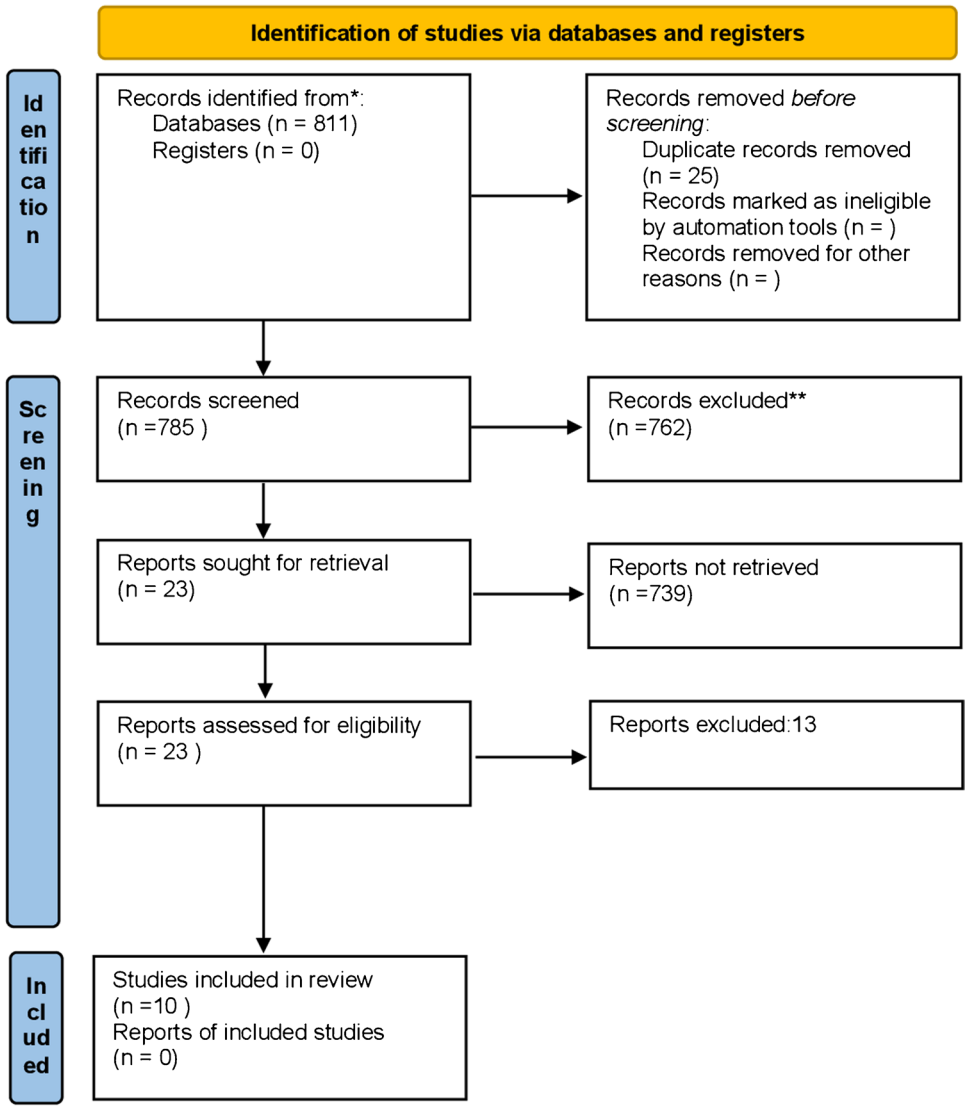


Fig. 1 Study selection process

Characteristics of the studies

The characteristics of the ten included studies are presented in Table 1. Of these ten studies, the majority (60%) were case–control studies [15, 23, 25, 27, 28, 30], while the remaining four studies (40%) were prospective studies [7, 24, 26, 29].

Of these, the vast majority (70%) were carried out in the Asian region; three studies (30%) were from India [23, 25, 28] and two studies each in Malaysia [15, 30] and Sri Lanka [7, 26]. Three single studies were in New Caledonia [27], Netherlands [24] and Slovenia [29]. Figure 2 presents the geographical distribution of the included studies. The number of participants in these studies varied widely from 50 [30] to 888 [7]. The publication years covered from 2011

Table 1 Main characteristic of the studies

First author, [ref #]	Year	Country	Study design	Total	TP	FP	FN	TN	Ref test
Niloofa (1) [7]	2015	Sri Lanka	Pro	888	288	119	56	425	ELISA
Niloofa (2) [7]	2015	Sri Lanka	Pro	868	286	121	76	405	MAT
Rao [15]	2019	Malaysia	CC	142	44	16	22	60	MAT
Panwala [23]	2011	India	CC	368	51	37	7	273	MAT
Goris (1) [24]	2013	Netherlands	Pro	375	16	10	22	327	MAT
Goris (2) [24]	2013	Netherlands	Pro	537	65	17	35	420	MAT
Goris (3) [24]	2013	Netherlands	Pro	350	63	5	24	258	MAT
Goris (4) [24]	2013	Netherlands	Pro	563	82	13	35	433	MAT
Bhatia (1) [25]	2015	India	CC	63	3	13	7	40	MAT
Bhatia (2) [25]	2015	India	CC	63	2	14	0	47	ELISA
Eugene (1) [26]	2015	Sri Lanka	Pro	84	34	12	6	32	MAT
Eugene (2) [26]	2015	Sri Lanka	Pro	84	31	15	8	30	EISA
Goarant [27]	2015	New Caledonia	CC	144	70	34	2	38	MAT
Panwala (1) [28]	2015	India	CC	100	73	1	7	19	ELISA
Panwala (2) [28]	2015	India	CC	100	24	45	4	27	MAT
Podgorsek [29]	2015	Slovenia	Pro	590	12	24	1	553	MAT
Alia [30]	2019	Malaysia	CC	50	9	6	10	25	MAT

Subset of data in a study; *CC*, case–control design; *ELISA*, enzyme-linked immunosorbent assay; *FN*, false negative; *FP*, false positive; *MAT*, microscopic agglutination test; *Pro*, prospective cohort design; *TF*, true negative; *TP*, true positive; *Ref #*, reference number; *Ref test*, Reference test

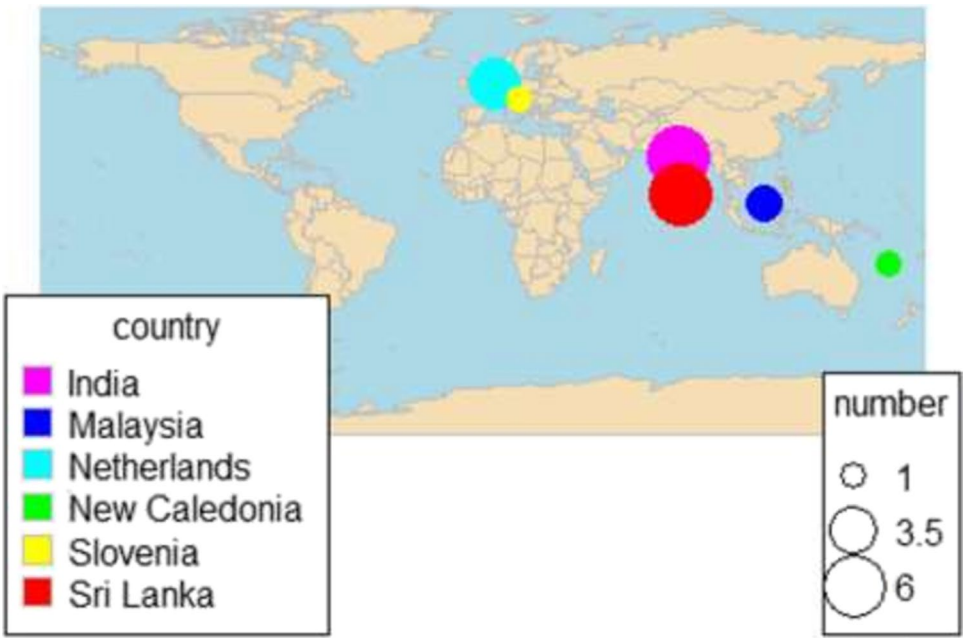


Fig. 2 Geographical distribution of the included studies

to 2019, and half (50%) was published in 2015 alone [7, 26–29].

QUADAS 2 — quality of the included studies

The methodological quality of individual study is shown in Additional File 3. Many studies included in this meta-analysis were with high or unsure risk of bias. The summary of the methodological quality assessment across all studies is provided in Additional file 4. The most frequent 40% of the included studies had high risk of bias in patient recruitments. All these studies were with unclear risk of bias in the selection of index test (100%) or the reference standard (100%). There were low concerns on the "applicability" of the included studies based on patient selection, index test and reference standard.

Test performances

Overall, the pooled sensitivity and specificity of Leptocheck-WB for the detection of leptospirosis using MAT as reference test were moderate at 0.75 (95% *CI*: 0.64 to 0.84, 10 studies, I^2: 85.9%) and 0.87 (95% *CI*: 0.77 to 0.94, 10 studies, I^2: 97.37%)), respectively. Of note is the substantial between-study heterogeneity (Fig. 3 and Additional file 5). Overall, the pooled sensitivity and specificity of Leptocheck-WB that used ELISA as reference test were also moderate at 0.85 (95% *CI*: 0.79 to 0.9, 4 studies, I^2: 27.49%) and 0.79 (95% *CI*: 0.71 to 0.85, 4 studies, I^2: 58.9%), respectively (Fig. 4 and Additional file 5).

DOR of Leptocheck-WB with MAT as reference test was 21 (95% *CI*: 10–44). When ELISA was the reference test, the DOR was 21 (95% *CI*: 9–46) (Table 2).

In general, specificity is better than the sensitivity, regardless of reference test. Between-study heterogeneity was relatively small for studies that used ELISA as reference test. This implied that the ability of Leptocheck-WB test was better in correctly classifying an individual as negative for leptospirosis. The results showed that the ability of Leptocheck-WB test to accurately identify a person as "diseased" (presence of leptospirosis) is 78%, range 66 to 87%. The ability of Leptocheck-WB test to accurately

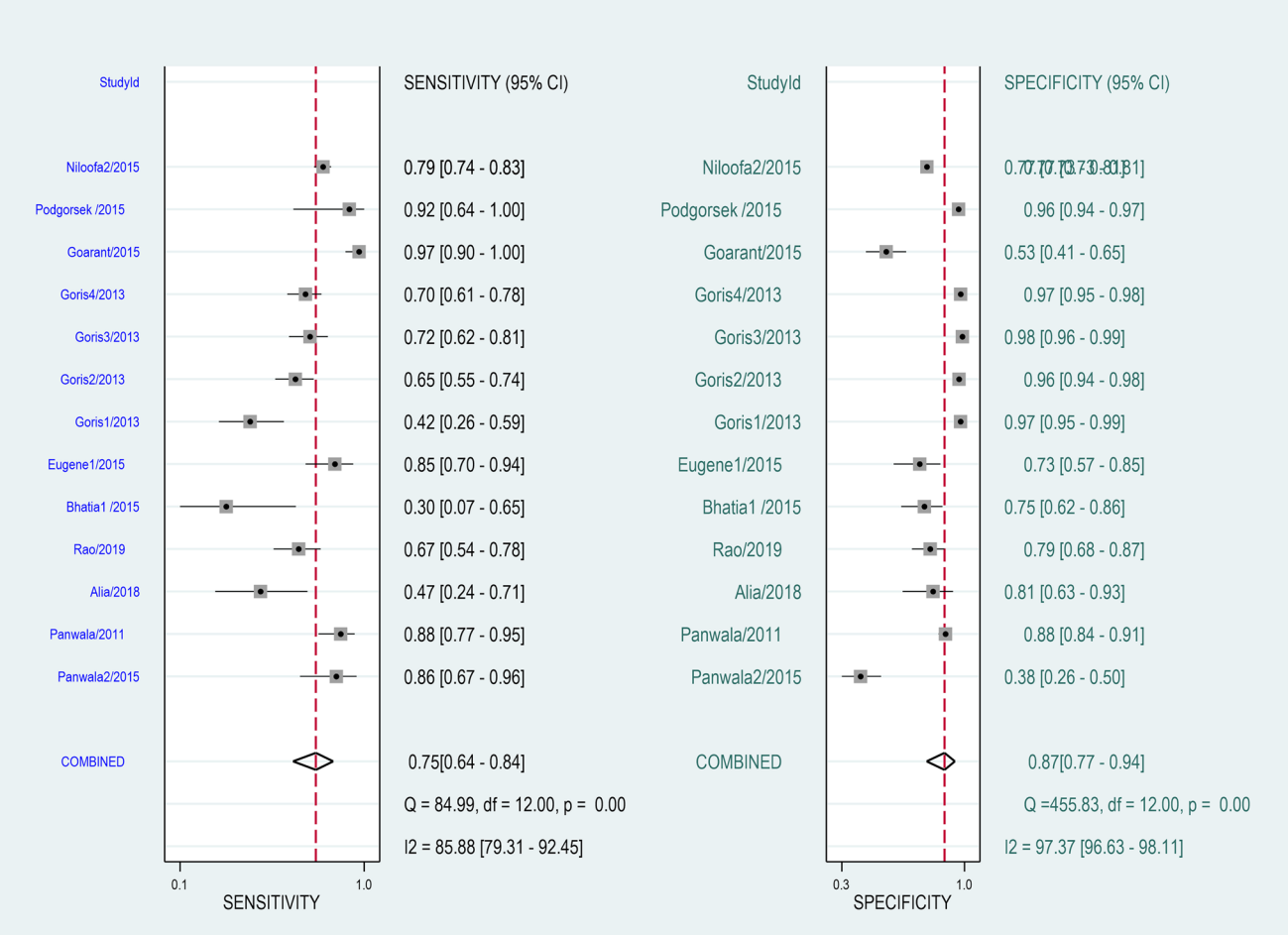


Fig. 3 Test performance with MAT reference test

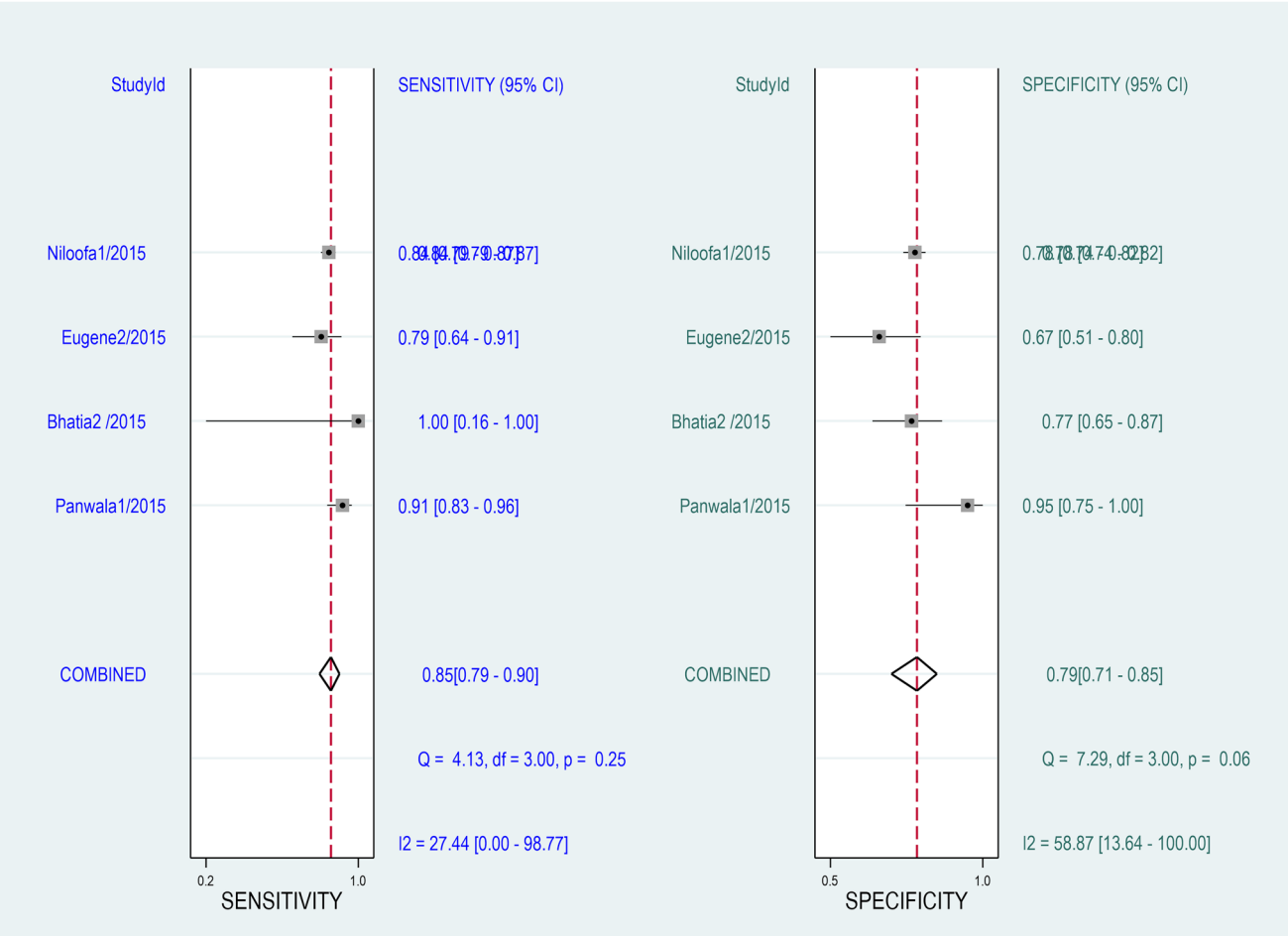


Fig. 4 Test performance with ELISA reference test

Table 2 Pooled diagnostic accuracy stratified by reference test

Reference Test	Indices	Estimate [95% *CI*]
MAT	Sensitivity	0.75 [0.64–0.84]
	Specificity	0.87 [0.77–0.94]
	Positive likelihood ratio	6.0 [3.3–11.1]
	Negative likelihood ratio	0.28 [0.19–0.41]
	Diagnostic odds ratio	21 [10–44]
ELISA	Sensitivity	0.85 [0.79–0.90]
	Specificity	0.79 [0.71–0.85]
	Positive likelihood ratio	4.0 [2.7–5.9]
	Negative likelihood ratio	0.19 [0.12–0.30]
	Diagnostic odds ratio	21 [9–46]

MAT, microscopic agglutination test

identify a person as disease free (absence of leptospirosis) is 84%, ranging from 74 to 90%. Of note was the substantial between-study heterogeneity as the I^2 values of the Leptocheck-WB test that used MAT were > 85% for both sensitivity and specificity (Fig. 3). Between-study heterogeneity was relatively lower in the Leptocheck-WB test that used ELISA (Fig. 4).

An SROC model for Leptocheck-WB test is presented in Figs. 5 and 6. The AUCs were 0.88 (95% *CI*, 0.85–91%) with MAT and 0.89 (95% *CI*, 0.86–91%) with ELISA. This indicated that the diagnostic performance of these tests was moderate to high accuracy. There was substantial between-study heterogeneity as the I^2 values of the Leptocheck-WB test were 93.7% for sensitivity and 96.6% for specificity (Fig. 4).

To investigate the source of heterogeneity, a meta-regression analysis with three covariates such as study design (i.e. prospective or not), sample size, risk of bias, patient recruitments for index test and the study design was performed. Of these potential confounding factors, risk of bias (p: 0.06) and study design (p: 0.09) had affected the sensitivity and specificity of the Leptocheck-WB test (Additional file 6).

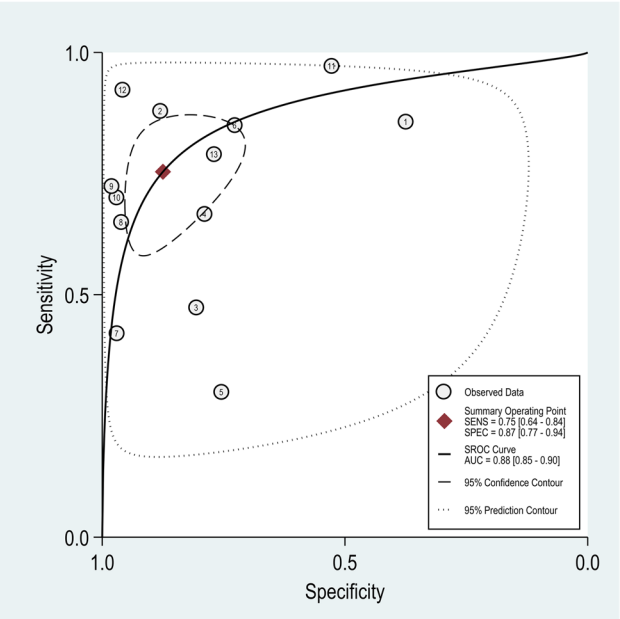


Fig. 5 SROC model with MAT reference test

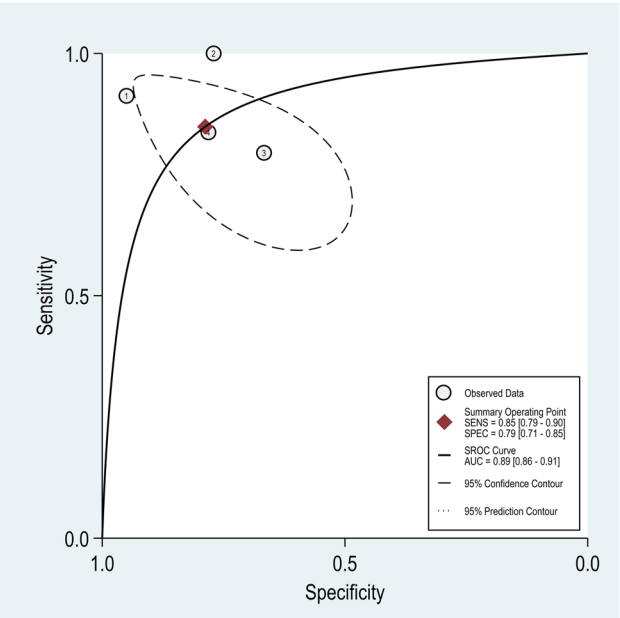


Fig. 6 SROC model with ELISA reference test

Discussion

The current meta-analysis study encompassed 10 studies from six endemic countries. The summary of findings is as follows:

1. All studies included were conducted in the endemic regions such as South Asia, Western Pacific and certain parts of Europe.
2. Compared with reference test MAT, the pooled sensitivities and specificities for Leptocheck-WB were 78% and 84%, respectively. This indicates Leptocheck-WB was better in ruling out acute leptospirosis than its confirmation.
3. Compared with reference test ELISA, the pooled sensitivities and specificities for Leptocheck-WB were 85% and 79%, respectively. This indicates Leptocheck-WB was better for detecting acute leptospirosis than in its confirmation.
4. Based on the AUC, the reference test ELISA (*AUC*, 0.89, 95% *CI*: 0.86–0.91) and MAT (*AUC*, 0.89, 95% *CI*: 0.86–0.91) could yield comparable diagnostic accuracy.
5. Methodological quality of studies and prospective or non-prospective nature of design were identified as the two main sources of between-study heterogeneity.

Leptocheck-WB has acceptable diagnostic accuracy, is simple to use, relatively inexpensive, easy to interpret, stable under extreme conditions with little or no processing and provides results within 1–2 h [31]. Of many RDTs for leptospirosis, Leptocheck-WB has a number of advantages. It can detect IgM antibodies occurring as early as 3–10 days after the onset of disease [29]. The current results showed that this Leptocheck-WB had less than perfect accuracy. This might be due to variation in population-related differences at the time of sample collections or the choice of reference test. A modelling study had highlighted that the true sensitivities of culture, MAT, and culture plus MAT are low. This is because *Leptospira* organisms are only present in the blood during the first week of untreated infection, and isolation of this bacterium from clinical samples is technically demanding [32]. This would be compounded if there was a substantial difference between suspected cases and probable leptospirosis cases tested. Moreover, this RDT can only detect IgM, and IgM titers are known to decline faster in patients [27]. Additionally, the test sensitivity depends on the causative leptospires. As there is a wide diversity of geographic distribution of most *Leptospira* serovars [24], the diagnostic accuracy with MAT will likely vary in different geographical locations.

Study limitations

We acknowledge limitations to the current study. Due to the limited number of studies with limited sample sizes, a low statistical power was an issue. Hence, there is the need for a larger study with greater statistical power [26]. There were several confounding factors that could have influenced the pooled accuracy of RDT. Due to paucity of data, we could

address only a few factors. Study design used in primary studies showed an impact on the accuracy of this RDT upon meta-regression analysis. This suggests difference in study design could affect the diagnostic accuracy of RDT for the detection of acute leptospirosis. There also is an inherent limitation of this RDT such as its genus-specific nature and inability to react and recognize the infecting serovar-specific IgM antibodies which could affect the reported sensitivity and specificity of Leptocheck-WB test [26]. The results of the test under evaluation must be compared with the results of the same samples characterized using a validated reference test defined as the gold standard [26, 29]. This is a case for the primary studies with case–control design, using samples from different status of infection in the two groups. Hence, the present findings should be interpreted with caution taking into account these concerns.

Implications

RDT Leptocheck-WB may be suitable as a PoC screening test for use in resource-limited settings. This is because of its acceptable sensitivity and specificity, rapid results within 15–20 min, ease of use and not requiring any special equipment [7]. However, due to rapid progression from acute to severe form of leptospirosis, it is not necessary to initiate treatment based on the results of RDT alone. Treatment with the appropriate antibiotic should be based on clinical and epidemiological suspicion in endemic settings [7, 27]. Leptospirosis is expected to become a globally important zoonotic disease due to rapid urbanization in developing countries along with slums areas and disaster such as floods [14]. Hence, the reliable and cost-efficient RDT is needed as PoC detection for early treatment. The choice of reference test affects the estimation of true accuracy of the index test as highlighted in published reviews [341]; statistical techniques that account for an imperfect reference standard will be an alternative methodological option [32]. Moreover, for leptospirosis diagnosis, an emerging test based on metal nanoparticle electrochemical biosensor is a promising candidate for the PoC [33].

Conclusions

The findings suggest that RDT Leptocheck-WB has an acceptable accuracy for the use of PoC screening of acute leptospirosis in endemic settings. Due to the limited number of studies and concerns over their methodological quality, further confirmation with large sampled, prospectively designed studies using the same samples for evaluating test accuracy is needed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-022-04420-9.

Acknowledgements The authors are grateful to the participants and researchers of the primary studies included in this review. We very much appreciated the anonymous reviewers and the editors for the comments and inputs provided. We thank our institutions for allowing us to perform this study.

Author contribution JWM: conceptualized, designed and interpreted; CN: designed, analysed and interpreted; SEH, NHH, WST: collected data; assisted in data analysis and interpretation; CN: wrote the first draft; JWM, SEH, NHH, WST: contributed additional information; JWM, CN: revised the manuscript; all authors read and approved the final manuscript.

Funding The International Medical University, Malaysia (ID: 353/2020).

Data availability All data generated or analysed during this study are included in this article and its supplementary information files.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

1. Levett PN (2001) Leptospirosis. Clin Microbiol Rev 14(2):296–326
2. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS et al (2015) Global morbidity and mortality of leptospirosis: a systematic review. PLoS Negl Trop Dis 9(9):e0003898
3. Victoriano AF, Smythe LD, Gloriani-Barzaga N, Cavinta LL, Kasai T, Limpakarnjanarat K et al (2009) Leptospirosis in the Asia Pacific region. BMC Infect Dis 4(9):147
4. Soo ZMP, Khan NA, Siddiqui R (2020) Leptospirosis: increasing importance in developing countries. Acta Trop. 201:105183
5. McBride AJ, Athanazio DA, Reis MG, Ko AI (2005) Leptospirosis. Curr Opin Infect Dis 18(5):376–386
6. Budihal SV, Perwez K (2014) Leptospirosis diagnosis: competancy of various laboratory tests. J Clin Diagn Res 8(1):199–202
7. Niloofa R, Fernando N, de Silva NL, Karunanayake L, Wickramasinghe H, Dikmadugoda N et al (2015) Diagnosis of leptospirosis: comparison between microscopic agglutination test, IgM-ELISA and IgM rapid immunochromatography test. PLoS One. 10(6):e0129236
8. WHO. (2003) Human leptospirosis: guidance for diagnosis, surveillance and control. WHO
9. Chirathaworn C, Inwattana R, Poovorawan Y, Suwancharoen D (2014) Interpretation of microscopic agglutination test for leptospirosis diagnosis and seroprevalence. Asian Pac J Trop Biomed 4(Suppl 1):S162–S164
10. Rosa MI, Reis MFD, Simon C, Dondossola E, Alexandre MC, Colonetti T et al (2017) IgM ELISA for leptospirosis diagnosis: a systematic review and meta-analysis. Cien Saude Colet 22(12):4001–4012

11. Lam J-Y, Low GK-K, Chee H-Y (2020) Diagnostic accuracy of genetic markers and nucleicacid techniques for the detection of *Leptospira* in clinical samples: a meta-analysis. PLoS Negl Trop Dis 14(2):e0008074
12. Lee JW, Park S, Kim SH, Christova I, Jacob P, Vanasco NB et al (2016) Clinical evaluation of rapid diagnostic test kit using the polysaccharide as a genus-specific diagnostic antigen for leptospirosis in Korea, Bulgaria, and Argentina. J Korean Med Sci 31(2):183–189
13. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA et al (2003) Peru-United States Leptospirosis Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3(12):757–771
14. Picardeau M (2013) Diagnosis and epidemiology of leptospirosis. Med Mal Infect 43(1):1–9
15. Rao M, Amran F, Aquilla N. (2019) Evaluation of a rapid kit for detection of IgM against *Leptospira* in human. Can J Infect Dis Med Microbiol. 5763595
16. McInnes MDF, Moher D, Thombs BD, McGrath TA, Bossuyt PM, The PRISMA-DTA Group et al (2018) Preferred Reporting Items for a Systematic Review and Meta-analysis of Diagnostic Test Accuracy studies: The PRISMA-DTA statement. JAMA 319(4):388–396
17. Bossuyt P, Davenport C, Deeks J, Hyde C, Leeflang M, Scholten R. (2013) Chapter 11: Interpreting results and drawing conclusions. In: Deeks JJ, Bossuyt PM, Gatsonis C (editors), Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy Version 0.9. The Cochrane Collaboration, http://srdta.cochrane.org/. Accessed 24 October 2021.
18. Leeflang MM (2014) Systematic reviews and meta-analyses of diagnostic test accuracy. Clin Microbiol Infect 20(2):105–113
19. Deshmukh H, Way SS (2019) Immunological basis for recurrent fetal loss and pregnancy complications. Annu Rev Pathol 14:185–210
20. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB et al (2011) QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med 155(8):529–536
21. Schueler S, Schuetz GM, Dewey M (2012) The revised QUADAS-2 tool. Ann Intern Med 156(4):323
22. Jones C, Athanasiou T (2005) Summary receiver operating characteristic curve analysis techniques in the evaluation of diagnostic tests. Ann Thorac Surg 79(1):16–20
23. Panwala T, Mulla S, Patel P (2011) Seroprevalence of leptospirosis in south Gujarat region by evaluating the two rapid commercial diagnostic kits against the mat test for detection of antibodies to Leptospira Interrogans. National J Comm Med 2(1):64–70
24. Goris MG, Leeflang MM, Loden M, Wagenaar JF, Klatser PR, Hartskeerl RA et al (2013) Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. PLoS Negl Trop Dis. 7(7):e2290
25. Bhatia M, Umapathy BL, Navaneeth BV (2015) An evaluation of dark field microscopy, culture and commercial serological kits in the diagnosis of leptospirosis. Indian J Med Microbiol 33(3):416–421
26. Eugene EJ, Handunnetti SM, Wickramasinghe SA, Kalugalage TL, Rodrigo C, Wickremesinghe H et al (2015) Evaluation of two immunodiagnostic tests for early rapid diagnosis of leptospirosis in Sri Lanka: a preliminary study. BMC Infect Dis 15:319
27. Goarant C, Bourhy P, D'Ortenzio E, Dartevelle S, Mauron C, Soupe-Gilbert ME et al (2013) Sensitivity and specificity of a new vertical flow rapid diagnostic test for the serodiagnosis of human leptospirosis. PLoS Negl Trop Dis. 7(6):e2289
28. Panwala T, Rajdev S, Mulla S (2015) To evaluate the different rapid screening tests for diagnosis of leptospirosis. J Clin Diagn Res 9(2):DC21-24
29. Podgorsek D, Cerar T, Logar M, Lesnicar G, Remec T, Baklan Z et al (2015) Evaluation of the immunochromatographic (Leptocheck) test for detection of specific antibodies against leptospires. Wien Klin Wochenschr 127(23–24):948–953
30. Alia SN, Joseph N, Philip N, Azhari NN, Garba B, Masri SN et al (2019) Diagnostic accuracy of rapid diagnostic tests for the early detection of leptospirosis. J Infect Public Health 12(2):263–269
31. TDR Diagnostics Evaluation Expert Panel, Banoo S, Bell D, Bossuyt P, Herring A, Mabey D et al (2010) Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Microbiol 8(12 Suppl):S17-29
32. Limmathurotsakul D, Turner EL, Wuthiekanun V, Thaipadungpanit J, Suputtamongkol Y, Chierakul W et al (2012) 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 55(3):322–331
33. Verma V, Kala D, Gupta S, Kumar H, Kaushal A, Kuča K et al (2021) Leptospira interrogans outer membrane protein-based nanohybrid sensor for the diagnosis of leptospirosis. Sensors (Basel) 21:2552

Evaluation of the immunochromatographic (Leptocheck) test for detection of specific antibodies against leptospires

Article *in* Wiener klinische Wochenschrift · September 2015

DOI: 10.1007/s00508-015-0853-0

CITATIONS
0

READS
158

8 authors, including:

Tjaša Cerar
University of Ljubljana
38 PUBLICATIONS **457** CITATIONS
SEE PROFILE

Mateja Logar
University of Ljubljana
33 PUBLICATIONS **1,010** CITATIONS
SEE PROFILE

Tatjana Remec
General Hospital Novo Mesto
4 PUBLICATIONS **8** CITATIONS
SEE PROFILE

Emil Pal
Slovenia Medical
12 PUBLICATIONS **32** CITATIONS
SEE PROFILE

original article

Wien Klin Wochenschr
DOI 10.1007/s00508-015-0853-0

Wiener klinische Wochenschrift
The Central European Journal of Medicine

Evaluation of the immunochromatographic (Leptocheck) test for detection of specific antibodies against leptospires

Daša Podgoršek · Tjaša Cerar · Mateja Logar · Gorazd Lešničar · Tatjana Remec · Zvonko Baklan · Emil Pal · Eva Ružić-Sabljić

Received: 17 February 2015 / Accepted: 17 August 2015

Summary
Background Leptospirosis is a febrile worldwide zoonosis. Routine diagnosis of leptospiral infection is based on demonstration of specific antibodies with serological tests. Performance of the reference serological test, the microscopic agglutination test (MAT), requires significant expertise. The aim of our study was to find out if leptospiral infection can be proven with simple, rapid, commercially available immunochromatographic Leptocheck test in order to introduce it for the first level diagnosis in emergency cases with less specialized laboratory staff.

Methods In all, 590 serum samples of patients with clinical manifestations suggestive of leptospirosis were collected and tested with MAT and Leptocheck test. For confirmation of the results some other diagnostic methods such as polymerase chain reaction (PCR) and Leptospira isolation were performed.

Results Results of both serological tests were consistent in 576/590 (97.63 %) cases but Leptocheck gave more positive results in comparison to MAT (36 and 12, respectively) at first patient's testing. Following up the patient, MAT became positive in majority of Leptocheck positive patients at first visit. Leptospiral DNA was detected in nine blood and six urine samples belonging to thirteen different patients while only two samples were culture positive.

Conclusion In comparison with serological tests, PCR and culture have low sensitivity. According to our findings we conclude that Leptocheck test can prove leptospiral infection and could be used for rapid diagnosis of leptospirosis, later the sample should be confirmed with MAT.

Keywords Leptospirosis · Diagnostics · Serological tests · Microagglutination test · Leptocheck test

univ. dipl. mikr. D. Podgoršek (✉) · T. Cerar · E. Ružić-Sabljić
Faculty of Medicine Ljubljana,
Institute of Microbiology and Immunology,
Zaloška 4,
1000 Ljubljana, Slovenia
e-mail: dasa.podgorsek@gmail.com

M. Logar
Department of Infectious Diseases,
University Medical Center Ljubljana,
Zaloška 2,
1000 Ljubljana, Slovenia

G. Lešničar
Department of Infectious Diseases, General Hospital Celje,
Oblakova 5,
3000 Celje, Slovenia

T. Remec
Department of Infectious Diseases, General Hospital Novo mesto,
Šmihelska cesta 1,
8000 Novo mesto, Slovenia

Z. Baklan
Department of Infectious Diseases,
University Medical Center Maribor,
Ljubljanska ulica 5,
2000 Maribor, Slovenia

E. Pal
Department of Infectious Diseases,
General Hospital Murska Sobota,
Ulica dr. Vrbnjaka 6,
9000 Murska Sobota, Slovenia

Introduction

Leptospirosis is a worldwide zoonosis caused by tightly coiled spirochetes with characteristic hooked ends belonging to the genus *Leptospira* [1]. In Slovenia, 0–30 cases of leptospiral infection are found every year, most of them in endemic area of Pomurje (the most eastern

Published online: 17 September 2015

part of Slovenia), where the risk of acquiring the disease is associated with occupational and recreational exposures [2]. Clinical presentation in humans varies considerably, ranging from mild influenza-like illness to jaundice, renal failure, bleeding, and sometimes also death [1–2]. Consequently the clinical diagnosis of leptospirosis is difficult, often inaccurate and can be confused with other febrile illnesses present in our geographic area such as hemorrhagic fever with renal syndrome caused by Hantaviruses [3]. There is evidence that three Hantaviruses (Hantaan, Puumala, and Dobrava) circulate simultaneously in Slovenia [4]. Routine diagnosis of leptospiral infection in Slovenia is based on demonstration of specific antibodies with serological tests as isolation of Leptospira from body fluids as well as molecular diagnosis is not always successful. Performance of the reference serological test, the microscopic agglutination test (MAT), is quite fastidious and time-consuming, requires significant expertise and the maintenance of stock cultures, involves the use of a battery of leptospires belonging to different serovars and uses live organisms, creating a risk of laboratory-acquired infection [5–6]. This is why simple serological tests which facilitate the rapid diagnosis of leptospirosis are welcome. Several alternative serological methods for antibody detection are available including the enzyme-linked immunosorbent assay (ELISA) for IgM and IgG antibodies, indirect hemagglutination assay (IHA), immunofluorescence, and microcapsule agglutination but all of the mentioned tests are requested to perform. Immunochromatographic tests (like Leptocheck) are rapid, qualitative, sandwich immunoassays for the detection of Leptospira specific IgM antibodies, the first immunoglobulin that appear after infection [7]. The broadly reactive genus specific antigen used in the tests allows the detection of Leptospira infections caused by wide range of strains of different serovars.

The aim of this study was to evaluate the immunochromatographic test for rapid diagnosis of leptospiral infection in order to introduce one for the first level diagnosis in emergency cases.

Patients, materials, and methods

Patients and samples. In the present study, 590 blood samples from patients with clinical manifestations suggestive for leptospirosis were tested with reference MAT and rapid Leptocheck test. Patients from different hospitals in Slovenia participated in the study. All blood samples were collected at the first patient's visit to the hospital and referred to the Institute of Microbiology and Immunology in Ljubljana. Blood was collected by venipuncture and was allowed to clot, after centrifugation serum was collected for analysis. For some patients additional serum samples two or more weeks after first testing as well as samples for isolation of the pathogen and molecular testing were available. Samples were obtained based mainly on clinicians' decision taking into account patient's clinical presentation and anamnesis.

For molecular diagnosis blood in ethylenediaminetetraacetic acid (EDTA), urine, and cerebrospinal fluid samples were obtained from patients and sent to the laboratory.

Whole-blood in hemoculture bottles and urine samples in sterile bottles were collected aseptically, and sent to the laboratory for Leptospira isolation.

MAT. The sera were examined by the MAT, using a panel of 13 serovars: Gryppotyphosa, Canicola, Sejroe, Pomona, Cynopteri, Copenhageni, Patoc, Australis, Autumnalis, Pyrogenes, Bataviae, Panama, and Javanica. The serum was diluted serially and live leptospiral antigen suspensions from a battery of 13 serovars endemic in our geographic area, were added and allowed to incubate at 37℃ for 1 hour. The serum/Leptospira culture mixtures were then examined by dark field microscopy for the presence of Leptospira agglutination or clearance, and titers were determined. Titers of ≥ 100 were considered positive [8].

Immunochromatographic test. Leptocheck (Zephyr Biomedicals, India) was performed following the manufacturer's instructions. The Leptocheck kit components were brought to room temperature before testing; 10 µl of serum was added into the sample port A and five drops of sample running buffer were dispensed in the buffer port B. At the end of 15 min, the results were read. Results were recorded as negative when only control band appeared. If the patient's result was positive, another colored band appeared in the test window.

Molecular diagnosis. Blood samples with EDTA were centrifuged at 800 rpm for 10 min, supernatant as well as urine samples were centrifuged at 14500 rpm for 30 min. Supernatant was removed, pallet was resuspended with 180 µl of MagNA Pure Bacteria Lysis Buffer (BLB) (Roche, Germany) and 20 µl of proteinase K and incubated for at least 10 min at 65 ℃ and for 10 min at 95 ℃. Total DNA was then extracted using automatic method on a MagnaPure Compact apparatus (Roche, Germany). For the molecular diagnosis Leptospira specific polymerase chain reaction (PCR) analyses were performed using two different PCR reactions targeting various sections of the *rrs* gene. The first one is classical PCR reaction amplifying DNA of both pathogenic and nonpathogenic Leptospira while the second one is nested PCR reaction with two set of primers amplifying DNA of pathogenic Leptospira only [9, 10].

Leptospira isolation and identification. Approximately 1 ml of each sample (whole blood or urine) was inoculated into tube containing 7 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium; more than one tube were inoculated per sample. Tubes were incubated at 28 ℃ for 9 weeks and examined for leptospiral growth once per week using dark field microscopy [1, 11, 12].

Isolated Leptospira strains were typed in a microtiter plate using serial dilutions of rabbit anti-*Leptospira* sera representing pathogenic and nonpathogenic serogroups. Equal volumes of culture and rabbit antiserum dilutions were mixed together, incubated at 37 °C for 1 h and examined for agglutination using dark-field microscopy [1].

Results

Results of MAT and Leptocheck test at first patient visit to the hospital are shown in Table 1; results of two tests were in concordance in 95.8 %.

At the first patient visit to the hospital 12/590 patients were MAT and Leptocheck positive (Table 1 and in Table 2, patient number 3, 5–6, 11, 17–18, 26–27, 29, and 36–38). Regarding MAT, titers ranged from 100 to 102,400 to serovars Semaranga, Australis, Sejroe, and Tarrasovi. Beside these 12 patients, there were 24 MAT negative/Leptocheck positive patients (Table 1 and in Table 2, patient number 1–2, 4, 7–10, 12–16, 19–20, 23–24, 28, 31–34, 39, 41, and 43) and 1 MAT positive/Leptocheck negative patient (Table 1 and in Table 2, patient no 42).

Within 2–4 weeks (median: 17 days) additional serum samples of 69/590 patients were sent to the laboratory.

Four of 12 MAT and Leptocheck positive patients at first testing (Table 1 and in Table 2, patient number 6, 11, 17, and 27) were sent to our laboratory for second testing and all stayed positive with both tests, while other 8/12 were not tested again serologically. Patient number 6, 17, and 27 received antibiotic therapy after first testing, while no data on antibiotic treatment is available for patient 11 (Table 2).

Twenty-one of 24 MAT negative/Leptocheck positive patients at first medical examination were also tested again while 3/24 were not tested for Leptospira—one of them died 5 h after hospitalization while others were not classified clinically as leptospiral infection and no additional tests were performed (Table 2, patient number 16, 19, and 41). At second serological testing, 16/21 described patients developed positivity with MAT sharing titers of 100–3200 to serovars Grippotyphosa, Canicola, Semaranga, Tarrasovi, Sejroe, Pomona, Icterohemorrhagiae, or Australis (Table 2, patient number 1–2, 7–10, 15, 20, 23–24, 28, 31–32, 34, 39, and 43). All these patients were Leptocheck positive at first medical examination and stayed Leptocheck positive at second testing, three of them were also PCR positive; leptospiral DNA was amplified from blood and/or urine samples of these patients (Table 2, patient number 2, 15, and 32). Other 5/21 patients who were MAT negative/Leptocheck positive at first visit stayed MAT negative/Leptocheck positive at second testing (Table 2, patient number 14) or became negative with both tests (Table 2, patient number 4, 12, 13, and 33). In the meantime Leptospira was isolated from blood sample of one of these five patients (Table 2, patient number 4). There was also a patient who was MAT positive/Leptocheck negative at first testing (Table 2, patient number 42) but no additional samples were sent to our laboratory. Data on antibiotic therapy for all mentioned patients is shown in Table 2.

Table 1 Comparison of the results of microscopic agglutination test (MAT) and immunochromatographic (Leptocheck) test for detection of leptospiral antibodies at patient's first visit to the hospital

MAT	Leptocheck		All
	Positive	Negative	
Positive	12 (2.03 %)	1 (0.17 %)	13 (2.20 %)
Negative	24 (4.07 %)	553 (93.73 %)	577 (97.80 %)
All	36 (6.10 %)	554 (93.90 %)	590 (100 %)

Among 590 patients, 4 patients that were MAT and Leptocheck negative at the first visit became MAT and Leptocheck positive at the second testing, sharing MAT titers of 3200–25,600 to serovars Australis, Semaranga, Cynopteri, and Icterohemorrhagiae (Table 2, patients number 21, 22, 30, and 35). Leptospiral DNA was isolated from blood sample of two of these four patients (Table 2, patients 30 and 35). In all, 2 from 590 patients that were MAT and Leptocheck negative at first testing within few weeks became MAT positive and remained Leptocheck negative (Table 2, patients 25 and 40); in one of these patients leptospiral DNA was confirmed in blood (Table 2, patient 25). Patients 21, 22, 25, 30, and 35 were under antibiotic therapy after first testing while no data on antibiotic treatment is available for patient number 40 (Table 2).

We also received second sera from 38/545 patients who were seronegative at first testing and all of them remained MAT and Leptocheck negative at further testing.

All other patients were not classified as leptospiral infection from clinical point of view and no additional samples were sent. However they were negative with both serological tests at first visit.

For confirmation of leptospiral infection, some clinicians followed up their patients and third patient's sample was sent to the laboratory. Within 2–4 weeks (median: 30 days) after second testing, we received third patient's sample from 13 patients, 4 of them that were MAT negative/Leptocheck positive (Table 2, patients 7, 9, and 34), and one MAT negative/Leptocheck negative (Table 2, patient 21) at the first visit, became MAT positive after second serological testing with titers 200–700 to serovars Semaranga, Panama, Australis, and Sejroe, and stayed MAT positive after third testing. After third testing 8/13 patients that were MAT negative/Leptocheck negative at first visit stayed negative with both tests.

Comparison of results after all testing with MAT and Leptocheck test is shown in the Fig. 1; it is evident that MAT test gave more positive results by weeks after infection (from 13 to 36 patients) while time after beginning of infection influenced less on Leptocheck test (from 36 to 43 patients); which means that during the early infection Leptocheck gave more positive results than MAT.

Molecular analyses were performed on 21 blood, 16 urine, and 1 cerebrospinal fluid sample of 29/45 patients who were either MAT positive or Leptocheck positive at

Table 2 Table includes results of patients who were positive at least once by one of two tests, microscopic agglutination test (MAT) or immunochromatographic (Leptocheck). Beside the results of MAT, and Leptocheck test for detection of leptospiral antibodies at patient's first, second, and third visit to the hospital table includes results of culture and molecular detection of leptospiral DNA (polymerase chain reaction, PCR) and data on antibiotic treatment at patient's first visit to the hospital

Patient	First testing		Second testing		Third testing		Other tests at first visit			Antibiotic treatment
	MAT	Leptocheck	MAT	Leptocheck	MAT	Leptocheck	PCR		Culture	
							Blood	Urine		
1	–	+	+	+	NT	NT	NT	NT	NT	Yes
2	–	+	+	+	NT	NT	+	+	–	Yes
3	+	+	NT	NT	NT	NT	NT	NT	NT	Yes
4[a]	–	+	–	–	NT	NT	–	–	+[a]	?
5	+	+	NT	NT	NT	NT	NT	NT	NT	?
6	+	+	+	+	NT	NT	NT	NT	NT	Yes
7	–	+	+	+	+	+	–	–	NT	Yes
8	–	+	+	+	NT	NT	–	–	NT	?
9	–	+	+	+	+	+	–	NT	NT	?
10	–	+	+	+	NT	NT	–	–	–	No
11	+	+	+	+	NT	NT	NT	NT	–	?
12	–	+	–	–	NT	NT	NT	NT	NT	No
13	–	+	–	–	NT	NT	NT	NT	NT	?
14	–	+	–	+	NT	NT	NT	NT	NT	No
15	–	+	+	+	–	+	–	+	NT	Yes
16	–	+	NT	NT	NT	NT	–	NT	NT	?
17	+	+	+	+	NT	NT	NT	–	NT	Yes
18	+	+	NT	NT	NT	NT	NT	–	NT	Yes
19	–	+	NT	NT	NT	NT	–	NT	NT	?
20	–	+	+	+	NT	NT	–	NT	NT	?
21	–	–	+	+	+	+	NT	–	–	Yes
22	–	–	+	+	NT	NT	–	NT	NT	Yes
23	–	+	+	+	NT	NT	NT	NT	NT	Yes
24	–	+	+	+	NT	NT	NT	–	NT	Yes
25	–	–	+	–	NT	NT	+	NT	NT	Yes
26	+	+	NT	NT	NT	NT	NT	NT	NT	?
27[b]	+	+	+	+	NT	NT	NT	–	+[b]	Yes
28	–	+	+	+	NT	NT	NT	NT	NT	?
29	+	+	NT	NT	NT	NT	–	NT	NT	?
30	–	–	+	+	NT	NT	+	NT	NT	Yes
31	–	+	+	+	NT	NT	NT	NT	NT	?
32	–	+	+	+	NT	NT	+	NT	–	Yes
33	–	+	–	–	NT	NT	NT	–	NT	No
34	–	+	+	+	+	+	NT	NT	NT	?
35	–	–	+	+	NT	NT	+	NT	–	Yes
36	+	+	NT	NT	NT	NT	+	+	NT	Yes
37	+	+	NT	NT	NT	NT	+	–	–	?
38	+	+	NT	NT	NT	NT	+	NT	NT	Yes
39	–	+	+	+	NT	NT	–	NT	NT	?
40	–	–	+	–	NT	NT	NT	NT	–	?
41	–	+	NT	NT	NT	NT	NT	NT	NT	Yes
42	+	–	NT	NT	NT	NT	NT	NT	NT	Yes
43	–	+	+	+	NT	NT	NT	NT	NT	?

? Data not available
NT Not tested
[a] *Leptospira* serovar Icterohemorrhagiae was isolated from patient's blood
[b] *Leptospira* serovar Gryppotyphosa was isolated from patient's blood

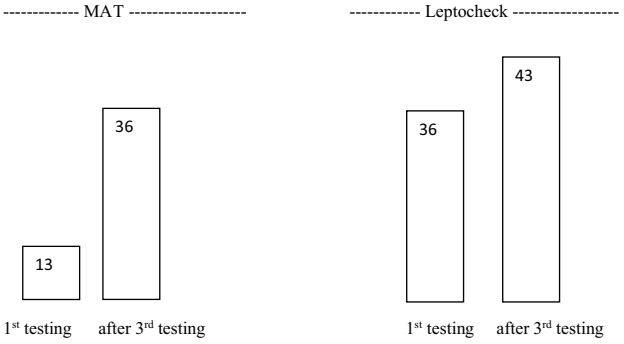


Fig. 1 Number of seropositive patients after first and third testing (4–8 weeks interval, median: 42 days) with microscopic agglutination test (MAT) and Leptocheck test

first visit. Leptospiral DNA was detected in eight blood (Table 2, patients 2, 25, 30, 32, and 35–38) and three urine (Table 2, patients 2, 15, and 36) samples obtained from nine patients.

For Leptospira isolation 10 samples belonging to 10/45 either MAT or Leptocheck positive patients were sent to our laboratory. Leptospira was isolated from 2/10 blood samples obtained from 1 MAT negative/Leptocheck positive patient and 1 MAT positive/Leptocheck positive patient at their first visit to hospital (Table 2, patient number 4 and 27). Isolated Leptospira were typed as *Leptospira* serovar Icterohemorrhagiae and Gryppotyphosa.

For patients who received other diagnosis and leptospirosis was clinically excluded no additional samples for molecular analysis or Leptospira isolation were sent to the laboratory.

Discussion

The diagnosis of leptospirosis is very complex, especially if clinical presentation does not suggest primary leptospiral infection. Clinical diagnosis of leptospiral infection in Slovenia could be confused with Hantavirus infection that is more frequently reported in Slovenia as well as with other infections, and clinical symptoms [4]. This is why rapid and accurate method for the diagnosis of leptospirosis is important for both clinician and patient. The reference serological test for confirming leptospirosis is MAT, which requires significant expertise, the maintenance of stock cultures and is time-consuming [6, 9]. Leptocheck is a rapid commercially available screening test for the diagnosis of acute leptospiral infection and was designed for countries with high incidence of leptospirosis. In this study, we wanted to assess the ability of the Leptocheck test to diagnose *Leptospira* infection by comparing the results of Leptocheck test with the results of the reference MAT test as well as other microbiological methods.

In Slovenia, the incidence of leptospirosis is relatively low, consequently there are not many positive samples [13] but as a part of diagnostic procedures for febrile illness with renal/hepatic involvement we perform more than 400 serological tests annually. In this study, samples belonging to 590 patients with clinical manifestations suggestive of leptospirosis were sent to our laboratory for serological testing but later majority of them received other diagnosis. Consequently, only 72 patients were tested again and only 13 samples were sent for third serological testing. This is also why we lost so many patients for molecular analysis and Leptospira isolation.

We found out that at first patient's presentation, the Leptocheck test was more sensitive than MAT, it detected more patients with Leptospira antibodies than MAT. Majority of these patients were followed up and checked with more caution (Table 2).

One of the advantages of the Leptocheck test is that Leptocheck detects IgM class antibodies and thus allows us to monitor the occurrence of the IgM antibodies, that may occur as early as 3–10 days after the onset of disease if antibiotic therapy was not given before, while MAT shows overall antibodies against Leptospira and is usually positive 10–12 days after the appearance of the first clinical symptoms and signs [2, 11]. The other advantages of Leptocheck test over MAT test are: simple performance which does not require significant expertise, results are known in 15 min and test could be performed in laboratories with less specialized equipment. The disadvantage of the Leptocheck test is that it does not have the ability to identify the strain that caused the infection.

Some other studies evaluating commercially available assays for detecting antibodies against Leptospira were reported in literature. Some of them showed poor diagnostic accuracy of evaluated tests [14, 15] but the results of the other studies are similar to ours [5, 6, 16–18]. We all found out that the use of the commercial fast tests could lead to rapid diagnosis of leptospirosis but the MAT still should be required to confirm serological results as well as to obtain information about serovar, which is important for epidemiological studies.

There are also other methods for the detection of leptospirosis. Culture is, for example, fastidious but valuable diagnostic method. Leptospira can be isolated from patient's blood, cerebrospinal fluid, and urine before antibiotic therapy is received [9, 11]. Culture plays a key role in the conformation of leptospiral infection and is very important for the epidemiological studies and for identifying virulence factors of Leptospira. But on the other hand, isolation of Leptospira from clinical samples is technically demanding, time-consuming, and has a low sensitivity. Furthermore, patients could be under antibiotic therapy before samples are taken which may lead to false-negative culture results. This is why culture is rarely used in clinical diagnosis [12]. During our study, we received 137 samples for Leptospira isolation and only two cultures were positive—*Leptospira* serovar Icterohemorrhagiae and *Leptospira* serovar Gryppotyphosa were isolated. This is due to low sensitivity, previous antibiotic therapy, and the fact that Leptospira is difficult to recover from clinical samples. One of two patients with positive culture was MAT negative/Leptocheck positive at first and second testing. Results are probably due to

the antibiotic therapy which can interrupt the synthesis of antibodies, and can result in further more difficult microbiological diagnosis of leptospirosis.

In recent years use of the molecular diagnostic techniques has increased. PCR is more sensitive and faster method for detection of Leptospira in clinical samples than culture and it also has an important role in the early detection of leptospirosis before antibodies appear. Disadvantages of molecular methods such as PCR are the complexity and high price, as well as the inability to identify serovar causing infection [9–10]. In our study, three PCR positive patients were negative with both tests and three were MAT negative/Leptocheck positive at first testing. All of them became MAT positive/Leptocheck positive at second presentation. This confirms how important molecular diagnosis is for early detection of leptospiral infection.

Because of the poor sensitivity of the culture and high rates of the molecular methods, serological diagnosis is still very important for leptospirosis confirmation. According to our finding, Leptocheck could be used as an alternative to MAT in emergency cases of suspected leptospirosis, but all results should be confirmed with a reference MAT which still remains very useful for identification of strains involved in infection, and for epidemiologic studies.

Ethical standards

The study approach was approved by the Medical Ethics Committee of the Ministry of Health of the Republic Slovenia (No. 122/05/12).

All procedures followed were in accordance with the ethical standards of the responsible committee (Medical Ethics Committee of the Ministry of Health of the Republic Slovenia) and with the Helsinki Declaration of 1975, as revised in 2008.z

Conflict of interest

The authors declare that there are no actual or potential conflicts of interest in relation to this article.

References

1. Faine S, et al., editors. *Leptospira* and leptospirosis. 2nd edn. Melbourne: MedSci; 1999.
2. Bedernjak J, editor. Leptospiroze pri nas in v svetu. Murska Sobota: Pomurska založba; 1993.
3. Palmer MF. Laboratory diagnosis of leptospirosis. Med Lab Sci. 1988;45:174–8.
4. Avsic-Zupanc T, et al. Isolation of a strain of a Hantaan virus from a fatal case of hemorrhagic fever with renal syndrome in Slovenia. Am J Trop Med Hyg. 1994;51:393–400.
5. Winslow WE, et al. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J Clin Microbiol. 1997;35:1938–42.
6. Levett PN, Whittington CU. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis. J Clin Microbiol. 1998;36:11–4.
7. Goris MG, Boer KR, Duarte TA, Kliffen SJ, Haartskerl RA. Human leptospirosis trends, the Netherlands, 1925–2008. Emerg Infect Dis. 2013;19:371–8.
8. Cole JR, Sulzer CR, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol. 1973;25:976–80.
9. Murry PR, Baron JE, Jorgensen JH, editors. Manual of clinical microbiology. 9th edn. Washington: ASM Press; 2007. pp. 963–70.Levett PN: Leptospira.
10. Ooteman MC, Vago AR, Koury MC. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J Microbiol Methods. 2006;65:247–57.
11. World Health Organisation. Human leptospirosis: guidance for diagnosis, surveillance and control. Geneva: International Leptospirosis Society; 2003.
12. Wuthiekanun V, et al. Optimization of culture of *Leptospira* from humans with leptospirosis. J Clin Microbiol. 2007;45:1363–5.
13. Institute of Public Health of the Republic of Slovenia. 2011 (Accessed 30 January 2012) http://www.ivz.si/gradiva_nalezljive_bolezni?pi=5&_5_Filename=4109.pdf&_5_MediaId=4109&_5_AutoResize=false&pl=105-5.3.
14. Blacksell SD, et al. Limited diagnostic capacities of two commercial assays for the detection of *Leptospira* Immunoglobulin M antibodies in Laos. Clin Vaccine Immunol. 2006;13:1166–9.
15. Desakorn V, et al. Accuracy of a commercial IgM ELISA for the diagnosis of human leptospirosis in Thailand. Am J Trop Med Hyg. 2012;86:524–7.
16. Levett PN, Branch SL. Evaluation of two enzyme-linked immunosorbent assay methods for detection of immunoglobulin M antibodies in acute leptospirosis. Am J Trop Med Hyg. 2002;66:745–8.
17. Zochowski WJ, Palmer MF, Coleman TJ. An evaluation of three commercial kits for use as screening methods for the detection of leptospiral antibodies in the UK. J Clin Pathol. 2001;54:25–30.
18. Smits HL. International multicenter evaluation of the clinical utility of a dipstick assay for detection of Leptospira—specific immunoglobulin M antibodies in human serum specimens. J Clin Microbiol. 1999;37:2904–9.

Eugene et al. BMC Infectious Diseases (2015) 15:319
DOI 10.1186/s12879-015-1080-z

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Evaluation of two immunodiagnostic tests for early rapid diagnosis of leptospirosis in Sri Lanka: a preliminary study

Egwin J. Eugene[1], Shiroma M. Handunnetti[1], Shalini A. Wickramasinghe[1], Thilini L. Kalugalage[1], Chathuraka Rodrigo[2], Hasith Wickremesinghe[3], Nandana Dikmadugoda[3], Pranitha Somaratne[4], H Janaka De Silva[5] and Senaka Rajapakse[2*]

Abstract

Background: Leptospirosis is often treated based on clinical diagnosis. There is a need for rapid laboratory diagnosis for this condition. The aim of this study was to compare the diagnostic accuracy of two rapid IgM based immunodiagnostic assays with the microscopic agglutination test (MAT), in acute leptospirosis infection.

Methods: MAT, IgM based immunochromatographic test (Leptocheck-WB) and IgM ELISA were performed using acute sera of patients clinically suspected to have leptospirosis ($n = 83$). Bayesian latent class modeling was used to compare the accuracy of these tests.

Results: Percentage positivity for MAT, Leptocheck-WB, and IgM ELISA were 48.1, 55.3, and 45.7 % respectively. Bayesian latent class modeling showed a combined positivity rate of leptospirosis of 44.7 %. The sensitivity of MAT, Leptocheck-WB and IgM ELISA were 91.4, 95 and 81.1 %, and specificity were 86.7, 76.4 and 83.1 %, respectively.

Conclusions: Leptocheck-WB has high sensitivity, and, because it is quick and easy to perform, would be a good screening test for acute leptospirosis infection. IgM ELISA has good specificity, and is comparable with MAT; given that it is easier to perform and more widely available than MAT, it would be a more appropriate confirmatory test for use in hospitals with limited access to a specialized laboratory.

Background

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species [1]. In Sri Lanka, disease notification data shows a steady increase in the incidence of leptospirosis over the last two decades, which is attributable to disease emergence as well as improved surveillance [2]. During a large outbreak which occurred in 2008, the reported incidence rate was 7099 cases (35.7 per 100,000 population), with 204 deaths. The disease continues to affect large numbers of people each year, especially those in the farming community, resulting in significant morbidity and economic impact. In 2013, there were 4308 reported cases, with 78 deaths, giving an epidemiological prevalence of 21.5 per 100,000 population.

Clinical features of leptospirosis are similar to dengue and many other tropical infectious diseases in Sri Lanka; thus early laboratory confirmation of the diagnosis will help guide clinicians to institute appropriate treatment early in the course of the illness and plan appropriate resource allocation, potentially preventing complications and death. In most countries, accurate laboratory diagnosis is a challenge. Microscopic agglutination test (MAT) is generally considered the standard immunological test used for diagnosis of leptospirosis. Laboratory confirmation based on MAT is a much delayed process, and it is technically challenging, requiring experience laboratory scientists to perform the tests, and the maintenance of live leptospira cultures. Often, treatment is initiated on the basis of clinical assessment with subsequent laboratory confirmation using MAT. There

* Correspondence: Senaka.ucfm@gmail.com
[2]Department of Clinical Medicine, Faculty of Medicine, University of Colombo, Colombo 08, Sri Lanka
Full list of author information is available at the end of the article

is a need for rapid diagnostic tests which can yield accurate results early on in the course of the clinical illness. Clinical leptospirosis is a biphasic illness, with a leptospiraemic phase occurring from the 4^{th} to 7^{th} day followed by a leptospiruric phase that can last for 4-30 days. Leptospiruria coincides with the immune phase which begins with the appearance of IgM antibodies [3, 4]. Therefore, IgM based serology is of diagnostic value during this phase. Many rapid serodiagnostic assays are available, such as IgM based microplate enzyme-linked immunosorbant assay (ELISA), IgM dot ELISA test, IgM dipstick, latex agglutination test and haemagglutination assay [5–8]. These rapid tests are easy to perform and read, although their scientific validity with respect to sensitivity and specificity need further evaluation. To date, only one study has been reported from Sri Lanka evaluating commercially available rapid immunodiagnostic kits [9]. This study evaluated the microplate ELISA (Institut Viron Serion GmbH, Germany) and showed very low sensitivity and specificity values. Other rapid serological diagnostic assays have not been evaluated against a reference test in Sri Lanka.

This study compared the efficacy of two rapid immunodiagnostic assays for the detection of leptospira IgM antibodies, *i.e.*, IgM based immunochromatography test (Leptocheck-WB test) and IgM based microplate ELISA, together with MAT. MAT detects both IgM and IgG antibodies. The validity of MAT as a gold standard could be considered imperfect, as MAT positivity may result from previous infection, while a MAT-negative result in the presence of positivity by an IgM-specific assay may occur due to either low sensitivity or serovar specificity of MAT. In settings such as this, Bayesian latent class modeling has been suggested to be a more suitable method for evaluating diagnostic tests, as it assumes that all tests are imperfect [7, 10]. We therefore compared these tests using Bayesian latent class modeling.

Methods

Patients

Serum samples were collected from patients clinically suspected to have acute leptospirosis, admitted to the National Hospital, Colombo, (NHSL) and Base Hospital, Homagama (BHH), Sri Lanka during the period June to September 2010. The following criteria, based on World Health Organisation-Leptospirosis Epidemiology Research Group (WHO-LERG) Epidemiological criteria were used to define a suspected case of leptospirosis; acute febrile illness with any of the following: headache, myalgia, arthralgia, conjunctival suffusion, meningeal irritation, anuria, oliguria, proteinuria, jaundice, hemorrhages, cardiac arrhythmia, skin rash; or with a contact history of exposure to water or soil contaminated with urine of infected animals. Patients with a clear alternative diagnosis were excluded. MAT, IgM Leptocheck-WB test, and ELISA were performed on acute serum samples from these patients. Informed written consent was obtained from all patients included in the study. There were 83 patients presenting with clinically suspected acute leptospirosis; mean age 39.9 years (SD ±15.2), male: female ratio was 20:1.

Laboratory methods

The Leptocheck-WB test kit was obtained from Zephyr Biomedicals, India, and the IgM ELISA Leptospira kit from Diagnostic Automation Inc., USA. MAT was performed at the Department of Bacteriology, Medical Research Institute, Sri Lanka. For MAT, sera which gave an agglutination of at least 50 % of the leptospires (compared with the control antigen) was considered to be positive, with a serum titre of ≥400 considered the positive threshold [11]. *Leptospira biflexa* serovar Patoc strain Patoc-1, which is an indicator strain, was used as antigen in all three tests, *i.e.*, MAT, Leptocheck-WB and IgM ELISA.

The Leptocheck-WB test kit components were used according to manufacturer's instructions. [13] Briefly, contents were placed at room temperature (RT; 25 °C), and the test device was labeled with the patient's identity (patient's code). Serum sample (10 μl) was added into the sample port twice and 5 drops of sample running buffer was dispensed into the buffer port immediately. The device was kept at RT and the results were read at the end of 15 min. Depending on various intensities observed, they were subjectively scored as 0, 1+, 2+, 3+ or 4+ based on the intensity of colour of the antigen band, using a colour reference diagram. A result of 1+ or greater was considered positive according to the manufacturers' instructions.

The antigen coated on the IgM ELISA kit used was whole cell lysate from *Leptospira biflexa* patoc-1. The IgM-ELISA kit was used according to manufacturer's instructions [10]. Briefly, 1:40 dilutions of serum samples were prepared using the dilution buffer provided with the ELISA kit. Rheumatoid factor (RF) absorbent (40 μl) was added to 100 μl of diluted test serum (patient sera and healthy control sera), mixed well, incubated in the tubes for 5 min and then added to ELISA plate. Pre-diluted negative and positive controls provided were added to the ELISA plate, and the RF absorbent added according to manufacturers' instructions. The plate was incubated at RT for 10 min. The contents were then washed 3 times with the diluted wash buffer provided, and two drops of enzyme conjugate were added to each well and incubated at RT for 10 min. The plate was washed 3 times with wash buffer and two drops of chromogen was added to each well and incubated at RT for 5 min. The reaction was stopped by adding 2 drops of the stop solution per well, mixed well, and the plate

was read at 450 nm using an ELISA plate reader (ELx800- universal microplate reader, Bio-Tek, Instruments INC, Canada.). The cut-off value was ≥ 0.848, calculated by plotting the Receiver Operating Characteristic (ROC) curve.

Statistical analysis

Data was entered and analyzed using SPSS® statistical software version 17. Descriptive analysis was done using percentages. The MICE tool (Modelling for Infectious Diseases Centre, Mahidol-Oxford Research Unit, Thailand [http://mice.tropmedres.ac/home.aspx]) was used for Bayesian latent class modeling. The use of Bayesian latent class models to determine the accuracy of diagnostic tests where the gold standard is imperfect has been described elsewhere [7].

Ethics statement

Ethics approval (Ref No. EC/09/054) was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Colombo.

Results

For the 83 patients included in the study, the positivity obtained by the three tests, *i.e.*, MAT, immunochromatography and IgM ELISA were 48.1, 55.3, and 45.7 % respectively. Positivity by all three tests was 32.5 %. The combined positivity for Leptocheck-WB and MAT was 41.0 % whereas positivity with Leptocheck-WB and IgM ELISA was 37.3 % and IgM ELISA and MAT was 34.9 % (Table 1).

Based on Bayesian latent class modeling, the combined positivity rate (*i.e.*, percentage positive based on any of the tests) of leptospirosis was 44.7 % (95 % Credible Interval [CrI] 30.8-60.8). The sensitivity of MAT, Leptocheck-WB and IgM ELISA were 91.4, 95 and 81.1 %, and specificity were 86.7, 76.4 and 83.1 %, respectively. Leptocheck-WB had the highest sensitivity, with a negative predictive value of 95.1 %. MAT still had the highest specificity, although the specificity of IgM ELISA was comparable (positive predictive values 84.7 and 79.6 %) (Table 2). Good convergence was also seen for all parameters by using the MICE tool (see methods). Bayesian p-values for the profiles ranged from 0.418-0.630, indicating good fitness.

Table 1 Detection of anti-leptospira antibodies in human sera, by MAT, Leptocheck-WB and IgM ELISA assays

Number of samples by test (%)	MAT	Leptocheck-WB	IgM ELISA
27 (32.5)	+	+	+
7 (8.4)	+	+	-
2 (2.4)	+	-	+
4 (4.8)	+	-	-
4 (4.8)	-	+	+
8 (9.6)	-	+	-
6 (7.2)	-	-	+
26 (31.3)	-	-	-
Total 83 (100.0)			
Positivity by single test	48.1 %	55.4 %	46.9 %

Discussion

The primary objective of this preliminary study was to compare the relative accuracy and suitability of two rapid immunodiagnostic tests, *i.e.*, an IgM based immuno-chromatographic test, *i.e.*, Leptocheck-WB, and a microplate IgM ELISA, together with the conventional MAT test which is used most widely for diagnosis currently.

Leptocheck-WB is easy to perform, is a rapid method which takes only 15 min, and requires only a single dilution with no requirement of special equipment. In contrast, IgM ELISA has several steps in its procedure with a time duration of about 50-60 min and requires an ELISA plate reader. Leptocheck-WB test gave consistent results and the bands were stable for more than 12 months. Both tests were relatively inexpensive with a cost of less than 200 SLR (1.6 USD/test). The positivity produced by Leptocheck-WB test was 55.3 % while that for IgM ELISA was 45.7 %. We suggest, based on our findings, that Leptocheck-WB would be an appropriate

Table 2 Comparison of diagnostic accuracy of MAT, Leptocheck-WB, and IgM ELISA, using Bayesian latent class modeling

Parameters	Bayesian latent class model % (95 % credible interval)
Combined positivity rate	44.7 (30.8 - 60.8)
MAT	
Sensitivity	91.4 (72.2 - 100)
Specificity	86.7 (70.7 - 99.3)
PPV	84.7 (63.6 - 99.3)
NPV	92.7 (71.2 - 100)
Leptocheck-WB	
Sensitivity	95.0 (79.3 - 100)
Specificity	76.4 (60.8 - 93.2)
PPV	76.5 (56.2 - 94.3)
NPV	95.1 (75.9 - 100)
IgM ELISA	
Sensitivity	81.1 (62.4 - 97.3)
Specificity	83.1 (68.8 - 94.8)
PPV	79.6 (60.4 - 94.4)
NPV	84.5 (64.0 - 98.2)

PPV positive predictive value, *NPV* negative predictive value

screening test for leptospirosis, especially in hospitals with limited laboratory facilities. Leptocheck-WB can easily be performed as an individual test, either with serum or whole blood samples, the assay contents are stable and could be transported and stored at ambient temperatures. The test is a portable package, and is easy to interpret, with no requirement for skills in handling specialized equipment such as an ELISA microplate reader. Previous studies have shown variable sensitivity and specificity of Leptocheck-WB. One study in South Gujarat showed Leptocheck-WB to have relatively low sensitivity (78.7) and higher specificity (88.3 %) [12], compared to the sensitivity and specificity of 95 and 76.5 % in our study. In contrast, another study, also in South Gujarat, showed Leptocheck-WB to have very high sensitivity (98.4 %), while specificity was similar to the previous study (86.9 %) [11].

Despite the high specificity and sensitivity shown with MAT, this test is more difficult to perform, requires a specialized laboratory and trained staff, and is thus not available routinely to clinicians in remote rural hospitals. Current WHO guidelines recommend a MAT titer of ≥400 in a single or paired serum sample or a 4-fold increase in MAT in acute and convalescent sera for laboratory conformation of Leptospirosis [13]. In our study, a MAT titer of ≥400 in acute serum was considered as the reference standard. However, MAT measures both IgG and IgM; the duration for which IgG and IgM levels persist after acute infection is not clearly known. Thus, MAT positivity could reflect previous rather than acute infection. Our own data suggests that IgM levels decrease rapidly 3 months after acute infection (unpublished data). We suggest that IgM ELISA would be a more suitable test than MAT for use by clinicians treating patients with acute infections, for the following reasons. Firstly, the fact that MAT is only available in larger laboratories leads to delays and difficulties in obtaining early results, while IgM ELISA is more likely to be available at the point of care. Secondly, there is mounting evidence that IgM ELISA maybe more sensitive in identifying infection early on in the course of illness, which will provide a definite advantage to the treating clinician to prioritize healthcare resources in a timely manner. A meta-analysis of studies of ELISA in leptospirosis showed that the pooled sensitivity for IgM ELISA tests was 80.4 (79.2–81.5), and specificity was 94.4 % (93.9–94.9) [14], values which are similar to our study. Nonetheless, there is considerable heterogeneity seen in ELISA tests for leptospirosis, in previous studies [14]. The fact that the commercial ELISA method used in our study has good sensitivity and specificity is of importance in the local context.

Arguably, the main limitation of these three tests is the fact that they are genus specific for *Leptospira biflexa.* There is considerable interest in developing serovar specific immunological tests, and also in-house immunodiagnostics which would give higher diagnostic specificity and sensitivity for local serovars. Nonetheless, our results show reasonably high accuracy with these commercial tests, making these pragmatic alternatives to guide clinicians treating patients with leptospirosis in resource limited developing country settings.

Interestingly, pooled meta-analysis shows that, based on ELISA, IgM antibodies are more specific that IgG antibodies for leptospirosis [14]. It is postulated that this could be due other febrile illnesses causing a non-specific rise in IgG, resulting in false positives. If this were the case, it would also suggest that MAT, which tests both IgG and IgM antibodies, is less specific than IgM. Nonetheless, MAT is likely to remain the epidemiological gold standard for diagnostic confirmation of leptospirosis infection, eventually to be replaced by genomic techniques.

This was a preliminary study with limited sample size, and our findings emphasize the need for a larger study with greater statistical power. Such a study is currently in progress by our research team. This study was limited to one season and emphasizes the need for coverage of different geographical regions of the country, a longer study period to include seasonal variations and a larger sample size. Factors that could affect the reported sensitivity and specificity of Leptocheck-WB test may include its genus specific nature and inability to react and recognize the infecting serovar specific IgM antibodies.

Conclusions

Our preliminary findings suggest that Leptocheck-WB would be a suitable screening test, and IgM ELISA an appropriate confirmatory test for patients with acute leptospirosis. MAT will remain the reference standard for epidemiological purposes, especially if PCR is not available, however it's availability is limited.

Abbreviations

MAT: Microscopic agglutination test; IgM: Immunoglobulin M; IgG: Immunoglobulin G; PCR: Polymerase chain reaction; ELISA: Enzyme-linked immunosorbant assay; RT: Room temperature; MICE: Modeling for infectious diseases centre; CrI: Credible interval.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SMH conceived the study. EJE, SAW, HJDeS, SR and SMH designed the study. CR, HW, ND and SR did the clinical assessment. EJE carried out the IgM ELISA and Leptocheck-WB assays. EJE, SAW and SMH analyzed and interpreted these findings. PS and TLK carried out and interpreted the MAT data. EJE wrote the first draft; SR made critical revisions; all authors critically revised the manuscript for intellectual content, read and approved the final version. EJE, SMH and SR are guarantors of the paper.

Acknowledgements
We thank the Director/Medical Superintendent, Consultants, Registrars, House Officers, Sisters & Nurses of National Hospital Sri Lanka and Base Hospital Homagama, Sri Lanka, Staff of Medical Research Institute, Dr Rohini Gunaratne, Dr Thamal Vithanage, and all patients & individuals who donated blood for this study. This work was supported by the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.

Author details
[1]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo 03, Sri Lanka. [2]Department of Clinical Medicine, Faculty of Medicine, University of Colombo, Colombo 08, Sri Lanka. [3]Base Hospital, Homagama, Colombo, Sri Lanka. [4]Department of Bacteriology, Medical Research Institute, Colombo 08, Sri Lanka. [5]Department of Medicine, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka.

Received: 7 February 2015 Accepted: 4 August 2015
Published online: 11 August 2015

References

1. Agampodi SB. Case definitions in Leptospirosis: a note to Sri Lankan researchers. Sri Lankan J Infect Dis. 2012;2(2):55–7.
2. Agampodi SB, Peacock SJ, Thevanesam V, Nugegoda DB, Smythe L, Thaipadungpanit J, et al. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. AmJTrop Med Hyg. 2011;85(3):471–8.
3. Bajani MD, Ashford DA, Bragg SL, Woods CW, Aye T, Spiegel RA, et al. Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. J Clin Microbiol. 2003;41(2):803–9.
4. Bal AM. Unusual clinical manifestations of leptospirosis. J Postgrad Med. 2005;51(3):179–83.
5. DebMandal M, Mandal S, Pal NK. Serologic evidence of human leptospirosis in and around Kolkata, India: a clinico-epidemiological study. Asian Pacific J Tropical Med. 2011;4(12):1001–6.
6. Effler PV, Bogard AK, Domen HY, Katz AR, Higa HY, Sasaki DM. Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. J Clin Microbiol. 2002;40(4):1464–9.
7. 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(3):322–31.
8. Gussenhoven GC, van der Hoorn MA, Goris MG, Terpstra WJ, Hartskeerl RA, Mol BW, et al. LEPTO dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human sera. J Clin Microbiol. 1997;35(1):92–7.
9. Agampodi SB, Thevanesam V, Senaratne T. Validity of a commercially available IgM ELISA test for diagnosing acute leptospirosis in high endemic districts of Sri Lanka. Sri Lanka J Infect Dis. 2014;4:83–9.
10. Limmathurotsakul D, Jamsen K, Arayawichanont A, Simpson JA, White LJ, Lee SJ, et al. Defining the true sensitivity of culture for the diagnosis of melioidosis using Bayesian latent class models. PLoS One. 2010;5, e12485.
11. Panwala T, Mulla S, Patel P. Seroprevalence of leptospirosis in south gujarat region by evaluating the two rapid commercial diagnostic kits against the mat test for detection of antibodies to leptospira interrogans. National J Comm Med. 2011;2:64–70.
12. Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LEPTO Dipstick: a rapid and simple method for serodiagnosis of acute leptospirosis. Trans R Soc Trop Med Hyg. 1999;93(2):161–4.
13. WHO. Report of the second meeting of the leptospirosis burden epidemiology reference group. Geneva, Switzerland: WHO; 2011.
14. Signorini ML, Lottersberger J, Tarabla HD, Vanasco NB. Enzyme-linked immunosorbent assay to diagnose human leptospirosis: a meta-analysis of the published literature. Epidemiol Infect. 2013;141(1):22–32.

Performance Evaluations

CE

ISO 13485:2016

AS A REFERENCE PRODUCT

LEPTOCHECK®-WB

Rapid test for the detection of IgM antibodies to Leptospirosis

Journal of Applied and Natural Science 6 (1): 12-13 (2014)

JANS

A study on few biochemical parameters of clinically suspected and laboratory confirmed *Leptospirosis* cases

D. B. Zala[1], Vikram Khan[2], and V. K. Das*

*Department of Medicine, Sri Vinoba Bhave Civil Hospital, Dadra & Nagar Haveli, Silvassa -396230, INDIA
[1]Department of Microbiology, DPHL, VBCH, Directorate of Medical & Health Services, Dadra & Nagar Haveli, Silvassa - 396230, INDIA
[2]Directorate of Medical & Health Services, Dadra & Nagar Haveli, Silvassa, 396230, INDIA
*Corresponding author. E-mail: vkdas511@gmail.com

Received: June 30,2012; Revised received: January 7, 2014; Accepted: January 30, 2014

Abstract: A study on biochemical parameters in clinically suspected and laboratory- confirmed leptospirosis cases was conducted in the Union Territory of Dadra and Nagar Haveli. Out of 50 clinically suspected samples of leptospirosis, 38% were positive from both ELISA IgM and rapid card test. In the present study, liver markers such as bilirubin, Serum Glutamate Oxalo acetic Transaminase (SGOT) and Serum Glutamate Pyruvic Transaminase (SGPT) encountered elevation 68%, 100% and 89% respectively in leptospiosis positive patent while 89% and 48 % of positive patients showed elevation in kidney marker, creatinine and blood urea. On the basis of liver and renal functions, a hospital can develop its own clinical algorithm to suspect the case of leptospirosis.

Keywords: Biochemical parameters, Immunochromatogaphy, SGOT, SGPT, Leptospirosis

INTRODUCTION

Leptospirosis is an infectious disease caused by pathogenic organisms belonging to the genus *Leptospira* that are transmitted directly or indirectly from animals to humans. Human-to-human transmission of leptospirosis occurs very rarely (Levett, 2001). The infection is commonly transmitted to humans by water contaminated by animal urine to come in contact with unhealed breaks in the skin, the eyes, or with the mucus membrane. Leptospira can cause wide range of clinical manifestations, from a mild, flue-like illness to a severe disease form, characterized by multi-organ system complications leading to death **(**WHO, 1999). Apart from humans, at least 160 mammalian species are infected like rats, cattle, pigs, buffaloes, horses, sheep, goats, squirrels, bandicoots and raccoons. It is most commonly found in tropical or subtropical countries and may be prevalent in both urban and rural regions. Most outbreaks of leptospirosis are reported in coastal regions: Gujarat, Mumbai, Kerala, Chennai and the Andaman Islands (Meenakshi *et al.*, 2009 and Sethi *et al.*, 2010). It is known that leptospirosis is widespread in farm and domestic animals in many parts of India (WHO, 1999), including the North-East, West Bengal, Bihar, Madhya Pradesh, Maharashtra, Andhra Pradesh, Karnataka, Tamil Nadu, Punjab and Haryana (Charoonruangrit and Boonpucknavig, 1964; Mamuthausethupathi *et al.*, 1995; Patel *et al.*, 2006; Sugunan *et al.*, 2009 and Velineni *et al.*, 2007). Every year in the season of monsoon, Surat, Navsari, Valsad and Dadra and Nagar Haveli regions of western India are mainly affected from the leptospirosis. The purpose of present investigation, to determine the involvement of liver and kidney in lepspirosis positive patient.

MATERIALS AND METHODS

Fifty Single or paired samples of clinically suspected leptospirosis patients were collected at Sri Vinoba Bhave Civil Hospital Silvassa during November 2011 to Janury 2012 and tested by Rapid card test working on the principle of immunochromatography, a unique two-site immunoassay on a membrane (Leptocheck Zypher Inc.). As the test sample flows through the membrane assembly of the test device, the anti-human IgM colloidal gold conjugate forms a complex with IgM antibodies in the sample. This complex moves further on the membrane to the test window 'T' where it is immobilized by the broadly reactive Leptospira genus specific antigen coated on the membrane, leading to the formation of a red to deep purple colored band at the test region. 'T' which confirms a positive test result. All rapid card screened samples were tested by IgM ELISA for confirmation. All ELISA IgM positive samples were considered as leptospirosis confirmed cases. All biochemical parameters of liver

Table 1. Showing abnormality in the function of liver and kidney in Leptospirosis positive patients.

Function	Parameters	Elevated value in Leptospirosis positive patient (%)
Liver	SGOT (U/L)	100
	SGPT (U/L)	89
	Bilirubin (mg/dL)	68
Kidney	Creatinine (mg/dL)	89
	Blood Urea (mg/dL)	49

and kidney was tested by fully automated biochemistry analyzer (Xpand Plus, Semens inc)

RESULTS AND DISCUSSION

Only thirty eight percent of the total clinical suspected cases were found positive for leptospirosis (n = 50). The maximum patient of leptospirosis was reported hepatic and renal dysfunction. The elevated indicator of abnormal liver function SGOT, SGPT and bilirubin ware encountered 100% 89% and 68 % in leptospirosis positive patient. In case of abnormality in the function of kidney, it was observed that 89% patients have elevated creatinine values and 49% patients have significantly increased values of blood urea (Table 1). It has also been observed that there were abnormal biochemical changes in both the liver as well as renal functions in 89% of laboratory confirmed cases. The value of abnormality in liver and renal function of laboratory negative cases was lesser in contrast of positive cases.

Leptospirosis is a major public health problem in tropical countries with potentially fatal systemic complications and multiorgan dysfunction, including hepatic and renal failure, with or without severe pulmonary hemorrhage syndrome (Levett *et al*., 2001). The abnormality due to leptospirosis in the liver and kidney have been reported time to time, In present investigation, the level of abnormality in liver and renal function in leptospirosis confirm cases corresponds with the studies conducted by Sethi *et al.* (2010), where 73% patients (63 out of 86) were with abnormality in the liver and kidney function. On other hand, only 35% patients were found with symptoms of abnormality in the liver and kidney by Prabhu *et al.* (2010), while only three patients (13%) had abnormality in liver and kidney function during an outbreak in Germany (Desai *et al.*, 2009).

Thus, it was concluded that the abnormal liver and renal function may be considered as an indicator of suspicious case of leptospirosis in highly endemic region and on the basis of elevation of abnormality in the liver and renal functions of suspected case, a hospital can develop their own clinical algorithm towards the confirmation of leptospirosis.

ACKNOWLEDGEMENT

We thank to the Mission Director (NRHM) UT of Dadra and Nagar Haveli Silvassa, and Integrated Disease Surveillance Programme for provide necessary support.

REFERENCES

Charoonruangrit, S. and Boonpucknavig, S. (1964). Leptospirosis at Chulalongkorn Hospital: A report of 54 cases. *J. of Medical Association. Thailand*, 47: 653-659.

Desai, S., van Treeck, U., Lierz, M., Espelage, W., Zota, L. and Sarbu, A. (2009). Resurgence of field fever in a temperate country: an epidemic of leptospirosis among seasonal strawberry harvesters in Germany in 2007. *Clinical Infectious Disease*, 48: 691-697.

Levett, P. N. (2001). Leptospirosis. *Clinical Microbioliology Reviews*, 14: 296-326.

Patel, B. K., Gandhi, S. J. and Desai, D. C. (2006). Clinico-epidemiological aspect of leptospirosis in South Gujarat. *Indian J. of medical Microbiology*, 24(4):322-325.

Mamuthausethupathi, Shivakumar, S., Suguna, R., Jaykumar, M. R., Vijayakumar R, Cor, E. and Carrington, D. G. (1995). Leptospiorosis in Madras – A clinical & seriological study (1990 – 91) *Journal of the Association of Physician India*, 43(7):456-458.

Meenakshi, M., Anuradha, De. and Dilip, T. (2009). *Leptospirosis* outbreak in 2005: L.T.M.G. hospital experience. *Indian Journal of Medical Microbiology*, 27 (2):153-155.

Prabhu, N., Joseph, P.I.D. and Chinnaswamy, P. (2010). Seroepidemiological trends of human leptospirosis in Coimbatore, India between 2007 and 2009. *Advances in Applied Science Research*, 1: 113-119.

Sethi, S., Sharma, N., Kakkar, N., Taneja, J., Chatterjee, S.S. and Banga, S.S. (2010). Increasing trends of leptospirosis in northern India: a clinicoepidemiological study. *PLOS Neglected Tropical Diseases*, 4: 579.

Sugunan, A. P., Vijayachari, P., Sharma, S., Roy, S., Manickam, P., Natarajaseenivasan, K., Gupte, M. D. and Sehgal, S. C. (2009). Risk factors associated with leptospirosis during an outbreak in Middle Andaman, India. *Indian Journal of Medical Research*, 130(1): 67-73.

Velineni, S., Asuthkar, S., Umabala, P., Lakshmi, V. and Sritharan, M. (2007). Serological evaluation of leptospirosis in Hyderabad, Andhra Pradesh: A retrospective hospital-based study. *Indian Journal of Medical Microbiology*, 25: 24-7.

WHO (1999). Leptospirosis worldwide, World Health Organization; Wkly. *Epidemiol. Rec*., 74:237-242.

Molecular characterisation and disease severity of leptospirosis in Sri Lanka

Kanchana Kumari Bandara[1,2], Manjula Weerasekera[1]/+, Chinthika P Gunasekara[1], Nilantha Ranasinghe[3], Chamil Marasinghe[4], Neluka Fernando[1]

[1]Department of Microbiology [4]Department of Medicine, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka
[2]Department of Basic Sciences, Faculty of Allied Health Sciences, General Sir John Kotelawala Defense University, Sri Lanka
[3]Base Hospital, Tangalle, Sri Lanka

Leptospirosis is a re-emerging zoonotic disease all over the world, important in tropical and subtropical areas. A majority of leptospirosis infected patients present as subclinical or mild disease while 5-10% may develop severe infection requiring hospitalisation and critical care. It is possible that several factors, such as the infecting serovar, level of leptospiraemia, host genetic factors and host immune response, may be important in predisposition towards severe disease. Different Leptospira *strains circulate in different geographical regions contributing to variable disease severity. Therefore, it is important to investigate the circulating strains at geographical locations during each outbreak for epidemiological studies and to support the clinical management of the patients. In this study immunochromatography, microscopic agglutination test and polymerase chain reaction were used to diagnose leptospirosis. Further restriction fragment length polymorphism and DNA sequencing methods were used to identify the circulating strains in two selected geographical regions of Sri Lanka.* Leptospira interrogans, Leptospira borgpetersenii *and* Leptospira kirschneri *strains were identified to be circulating in western and southern provinces.* L. interrogans *was the predominant species circulating in western and southern provinces in 2013 and its presence was mainly associated with renal failure.*

Key words: *Leptospira* - molecular characterisation - Sri Lanka

Leptospirosis is an endemic, zoonotic disease of public health importance in Sri Lanka (Victoriano et al. 2009). Seasonal outbreaks of leptospirosis occur annually and in 2013, 4,276 cases were reported to the Epidemiological Unit of Sri Lanka. Since Sri Lanka is predominately an agricultural country with a heavy rain fall, exposure to *Leptospira* is a major occupational hazard (Brenner et al. 1999). *Leptospira interrogans*, *Leptospira santarosai*, *Leptospira kirschneri*, *Leptospira borgpetersenii* and *Leptospira weilli* have been reported from several geographical locations in Sri Lanka at different time periods with varying disease severity (Brenner et al. 1999, Agampodi et al. 2012, 2014, Nwafor-Okoli et al. 2012).

Due to the highly endemic nature and associated morbidity and mortality of this disease, it is important to investigate the circulating strains at geographical locations during each outbreak for epidemiological studies and to support the clinical management of the patients.

SUBJECTS, MATERIALS AND METHODS

This was a prospective hospital based study in western and southern provinces in Sri Lanka between January 2013-January 2014. All the patients more than 18 years of age, presenting with clinically suspected leptospirosis according to the World Health Organization (WHO) guideline admitted to the medical wards were included in the study.

Informed consent was obtained from all suspected patients and sociodemographic data and risk factors were gathered using a pre-tested interviewer administered questionnaire. A venous blood sample of 5 mL was collected following standard procedures and aliquoted into a plain tube for serum separation and the rest added to an ethylenediamine tetraacetic acid (EDTA) tube for DNA extraction. All samples were transported at 4ºC to the Department of Microbiology, University of Sri Jayewardenepura, Sri Lanka.

IgM immunochromatographic assay and microscopic agglutination test (MAT) - Leptospira infection was presumptively diagnosed by detecting *Leptospira* specific IgM using a rapid immunochromatographic assay kit (Leptocheck WB; Zephyr Biomedicals, India) following the manufacturer's instructions. MAT was done in order to obtain single MAT antibody titres using the genus specific *Leptospira biflexa* serovar Patoc 1 strain (Medical Research Institute, Sri Lanka) and ≥ 400 titre was considered as positive for MAT (WHO 2010).

DNA extraction - EDTA blood samples (200 µL) were used for *Leptospira* DNA extraction using QIAamp DNA blood mini kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Eluted DNA was quantified and purity was checked using Nanodrop 2000/200C spectrophotometer (Thermo Fisher Scientific, USA).

FlaB polymerase chain reaction (PCR) assay - PCR assay was used to amplify flagella gene present in pathogenic *Leptospira* species (Kawabata et al. 2001, Natara-

doi: 10.1590/0074-02760150070
Financial support: World Class University Project (PhD/01/2012), University of Sri Jayewardenepura
+ Corresponding author: mmweera@yahoo.com
Received 18 February 2015
Accepted 19 May 2015

jaseenivasan et al. 2012). Amplification of isolated DNA was carried out in 50 µL volume with 0.5 µL template DNA, 5 µL 5X green GoTaq® Flexi buffer (pH 8.5) (Promega, USA), 2 mM $MgCl_2$ (Promega), 0.1 µM of each primer (F1-TCTCACCGTTCTCTAAAGTTCAAC, R1-CTGAATTCGGTTTCATATTTGCC), 0.4 mM deoxy nucleotide triphosphate (dNTP) mix (Promega) and 0.25 units of Taq DNA polymerase (Promega). *L. interrogans* DNA was used as a positive control and a negative control without the template DNA were included in each PCR assay. PCR amplification was initiated at 94ºC for 5 min followed by 45 cycles of 94ºC for 1 min, 56ºC for 1 min, 72ºC for 90 s and a final elongation step at 72ºC for 10 min with final hold at 4ºC. The resulting amplicon was 793 bp and these were stored at 4ºC until further analysis.

Restriction fragment length polymorphism (RFLP) - PCR products of flaB PCR positive patient samples were used for RFLP digestion using Hae III and Hind III restriction enzymes (Kawabata et al. 2001). The restriction digestion was carried out in 20 µL of volume in a sterile microcentrifuge tube. The reaction mixture contained 10 µL of PCR product, 2 µL of 10 X RE buffer (Multicore™ buffer, Promega), 0.5 µL restriction enzyme (10 U/µL), 0.2 µL of acetylated bovine serum albumin (10 µg/µL) and distilled water to a final volume of 20 µL. The reaction mixture was incubated in an incubator at 37ºC for 5 h. The final product was subjected to electrophoresis using 2% agarose gel in tris-acetate-EDTA buffer containing 5 µg/mL ethidium bromide (Sigma Aldrich). Each digested PCR product was mixed with 1/5 volume of the gel loading buffer (Promega) and loaded into the agarose gel. Electrophoresis was carried out at room temperature for one and half hours. At the end of the electrophoresis the gel was visualised under ultraviolet transilluminator (Biometra GmbH, Germany). RFLP was done with three reference serovars: *L. interrogans* serovar Canicola, Icterohaemorrhagiae and Pyrogenes. An undigested PCR product, where the reaction mix was prepared without Hind 111, Hae 111 restriction enzymes, was used as a control (Figs 1, 2, Lane 2).

Nested PCR - A single tube nested PCR was used to amplify 16S rDNA gene specific for pathogenic and intermediate *Leptospira* species. Amplification was carried out using PCR primers: rrs-outer F (5^1-CTCAGAACTAACGCTGGCGGCGCG-3^1), rrs-outer-R (5^1-GGTTCGTTACTGAGGGTTAAAACCCCC-3^1), rrs-inner-F (5^1-CTGGCGGCGCG T CTTA-3^1), rrs-inner-R (5^1-GTTTTCACACCTGACTTACA-3^1) (Boonsilp et al. 2011). PCR master mix consisting of 0.5 µL template DNA, 5 µL 5X green GoTaq® Flexi buffer (pH 8.5) (Promega), 4 mM $MgCl_2$ (Promega), 0.2 pmol of each outer primer, 1.2 pmol of inner F, 5 pmol of inner R, 0.2 mM dNTP mix (Promega) and 0.25 units of Taq DNA polymerase (Promega) were used in a total volume of 25 µL. PCR reaction was carried out using a thermal cycler (Techne Flexigene, UK) with an initial denaturation at 95ºC for 2 min followed by 40 cycles of 95ºC for 10 s, 67ºC for 15 s, 72ºC for 30 s, another 40 cycles of 95ºC for 10 s, 55ºC for 15 s, 72ºC for 30 s and a final elongation step at 72ºC for 10 min. The resulting amplicon size was a 547 bp. Amplicons were visualised by gel electrophoresis using an 1.5% agarose gel. *L. interrogans* Serovar Canicola and *Leptostpira fainei* BUT 6 strain were taken as positive controls and *L. biflexa* Patoc 1 strain and no template control were used as the negative controls.

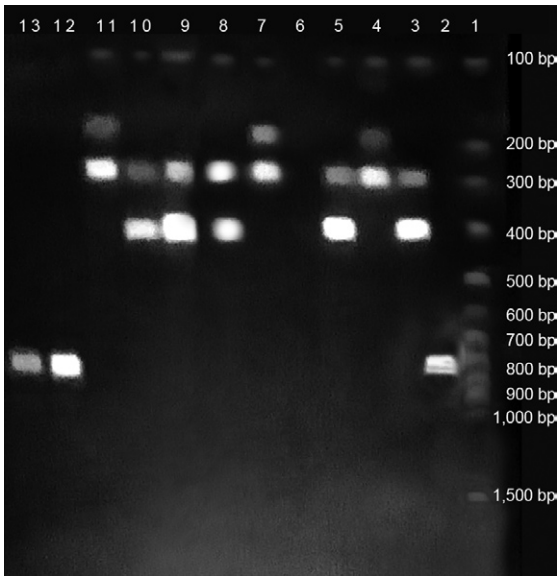


Fig. 1: hae 111 digestion of *Leptospira*. Lane 1: 100 bp DNA marker; 2: undigested polymerase chain reaction (PCR) product; 3: *Leptospira interrogans* serovar Canicola (100 bp, 300 bp, 400 bp); 4: *L. interrogans* serovar Icterohaemorrhagiae (100 bp, 200 bp, 300 bp); 5: *L. interrogans* serovar Pyrogenes (100 bp, 300 bp, 400 bp); 6; *Leptospira biflexa* Patoc 1 strain; 7-13: flaB PCR positive patient samples.

PCR products were purified using a PCR product purification kit (Promega) according to manufacturer's protocol and sequenced bidirectionally at Macrogen Inc (South Korea). DNA sequences were obtained using 3.1 Big Dye chemistry. Individual gene sequences were aligned using Bio Edit v.7.0.9.0. Consensus sequences were generated using Chromas v.5.0 and species were identified using National Center for Biotechnology Information (NCBI) BLAST. The gene sequences were deposited in the NCBI GenBank and accessions were obtained. Phylogenetic tree was developed using MEGA 6.0 (Fig. 3).

Ethics - Ethical approval was granted from the Ethical Review Committee of University of Sri Jayewardenepura (application 702/12).

RESULTS

Out of the 168 leptospirosis suspected patients 153 (91%) were males while 15 were females. Of these, 43.1% were farmers, 22.4% were outdoor laborers, 12.5% were indoor domestic workers and others included indoor office workers, housewives and school students. The mean age of the study sample was 41 years (± 20). The median duration of fever on admission was six days (± 2.5). Thirty-nine patients (23%) had been treated with antibiotics before admission to the hospital. Leptocheck rapid immunochromatographic assay for *Leptospira* IgM were

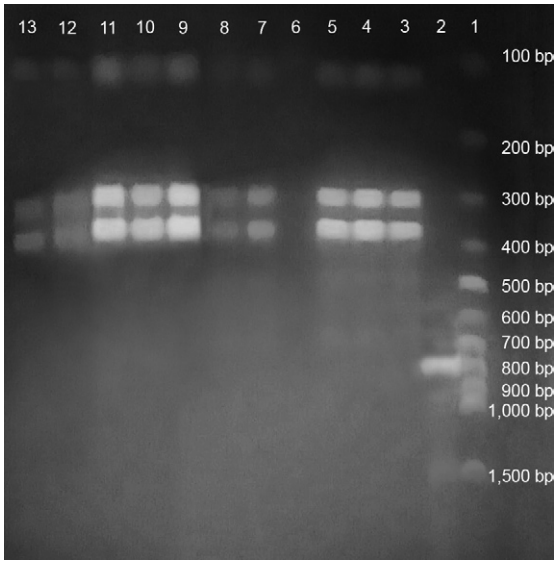


Fig. 2: hind 111 digestion of *Leptospira*. Lane 1: 100 bp DNA marker; 2: undigested polymerase chain reaction (PCR) product; 3: *Leptospira interrogans* serovar Canicola; 4: *L. interrogans* serovar Icterohaemorrhagiae; 5: *L. interrogans* serovar Pyrogenes; 6: *Leptospira biflexa* Patoc 1 strain; 7-13: flaB PCR positive patient samples.

TABLE I

Results of the laboratory diagnosis of leptospirosis based on microscopic agglutination test (MAT)[a], polymerase chain reaction (PCR) and immunochromatographic assay (Leptocheck) identification methods

Category (*Leptospira* case definition)	Method	Result	Patients n (%)
Definitive cases	MAT	+	61 (36)
	PCR	+	14 (8.3)
	MAT and PCR	+	7 (4.2)
	MAT or PCR	+	66 (39.2)
	MAT, PCR and Leptocheck	+	6 (3.6)
Presumptive cases	Leptocheck	+	84 (50)
Unconfirmed cases	MAT, PCR and Leptocheck	-	73 (43.4)
Total	-	-	168 (100)

a: single sample MAT ≥ 1:400; -: negative; +: positive.

positive in 84 (50%) while 13 (7.7%) were positive by flaB PCR. Of the 168 suspected patients, 61 (36%) had MAT titre of ≥ 1:400 (Table I) among them, 90% had a MAT titre of ≥ 800.

When the flaB PCR products were subjected to restriction enzyme digestion by Hae III, the DNA of reference strains, *L. interrogans* serovar Canicola and Pyrogenes (Fig. 1, Lanes 3, 5) resulted in three bands (100 bp, 300 bp and 400 bp). When the patient samples were tested by digestion with Hae III, three patients (Fig. 1, Lanes 8-10) had a restriction digestion pattern corresponding to *L. interrogans* serovar Canicola or Pyrogenes. Hae III restriction digestion was not able to differentiate between serovars Canicola and Pyrogenes. The reference DNA from *L. interrogans* serovar Icterohaemorrhagiae (Fig. 1, Lane 4) resulted in 3 bands (100 bp, 200 bp and 300 bp). Two patients in our study had a similar RFLP pattern corresponding to serovar Icterohaemorrhagiae (Fig. 1, Lanes 7, 11). A single band of 700 bp was observed in two patients (Fig. 1, Lanes 12, 13) and they were identified as *L. borgpetersenii* by DNA sequencing.

Hind 111 digestion resulted in three DNA fragments 100 bp, 300 bp and 350 bp in all reference strains; *L. interrogans* serovar Canicola, Icterrohaemorrgiae and Pyrogenes. All patient samples tested gave the same banding pattern (Fig. 2). Therefore Hind III was found to be less discriminative in the identification of *Leptospira* serovars.

Of the 84 *Leptospira* IgM positive patients, 12 were confirmed as leptospirosis using the nested PCR targeting the 16S rDNA gene. Interestingly, two IgM negative patients also gave positive results by rrs PCR. Therefore, 14 patients had confirmed leptospirosis by rrs PCR.

When risk factors were considered among the 14 leptospirosis confirmed patients, being a farmer ($p = 0.017$), outdoor laborer ($p = 0.046$) and contact with contaminated water ($p = 0.007$) showed a significant association with having leptospirosis. All the confirmed leptospirosis patients had an exposure history prior to the onset of the disease. Of these, nine patients reported exposure to contaminated water sources (paddy/agricultural land and flood), five reported animal exposure (cattle, rats and dogs) and three had either cracked heels or wounds on their feet.

Based on sequence analysis, *L. interrogans* was the most common cause of disease in this study (n = 11, 78.57%) followed by *L. borgpetersenii* (n = 2, 14.28%) and *L. kirschneri* (n = 1, 7.14%). The consensus sequences were submitted to GenBank and accessions were obtained as shown in Table II. A BLAST search revealed 99-100% identity of our isolates to *L. interrogans*, *L. borgpetersenii* and *L. kirschneri* (Table II).

Phylogenetic analysis shows that *L. interrogans* strains in our study were similar to the *Leptospira* identified in the 2008 outbreak in the central province of Sri Lanka (Fig. 3). Specimens SLUSJ_1, 2, 16, 111, 160 and 181 in our study were identified as *L. interrogans* which were closely related to isolate 68-JF910147 identified in the 2008 outbreak while specimen SLUSJ_3, 4, 19, 23 and 119 were closely related to *L. interrogans* isolate 229-JF910145 and isolate 109-JF910144 which were also identified during this outbreak (Agampodi et al. 2011). Specimen SLUSJ_12 and 70 were identified as *L. borgpetersenii* and specimen SLUSJ_176 was identified as *L. kirschneri* strains (Table II).

When clinical symptoms were analysed almost all patients were febrile on admission and had prostration. Headache (57%), myalgia (57%) and muscle tenderness (43%) were the common symptoms found in all confirmed cases. Conjunctival haemorrhage was seen in 35.7% of the confirmed leptospirosis patients. Elevated blood urea was seen in 14.2% whilst serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase were

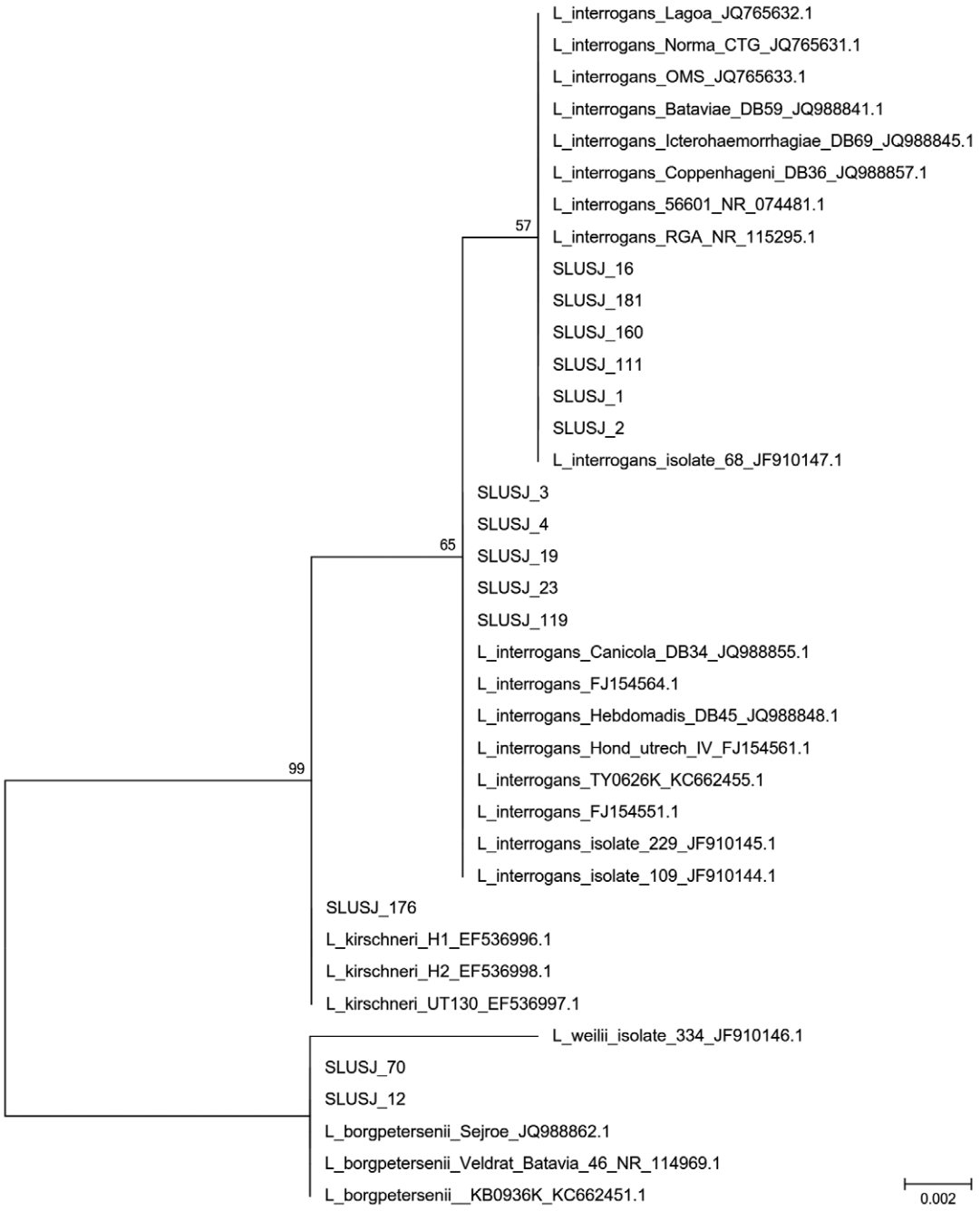


Fig. 3: phylogenic analyses were conducted with MEGA 6.0, the phylogenetic tree being drawn based on 1,000 bootstrap replicates with Kimura 2-parameter. The numbers on the nodes are the bootstrap support after 1,000 replicates. The specimens identified in the study are denoted by SLUSJ_ 1, 2, 3, 4, 12, 16, 19, 23, 70, 111, 119, 160, 176 and 181.

raised in 28.5% patients. Of these patients, 35.7% had leucocytosis and 57.5% had neutrophilia whilst haematuria (> 5 red blood cells per high power field) was seen in 35.7%. Serum creatinine levels were elevated in 7.14%. Electrocardiography changes were seen in 14.2%. Among the leptospirosis confirmed patients 28.5% required ICU treatment. Of these patients, 75% had infection due to *L. interrogans* and 25% had *L. borgpetersenii* infection. Renal failure was seen in 35.7% of the confirmed cases out of them, 80% were due to *L. interrogans*.

DISCUSSION

Leptospirosis is a widespread zoonotic infection gaining rapid importance in Sri Lanka due to the fact that the disease is associated with high morbidity and mortality (Agampodi et al. 2011, 2014, Nwafor-Okoli et al. 2012). In this study population, 50% were presumptively identified as leptospirosis, whilst 36% were confirmed by MAT (titre ≥ 400) (WHO 2010) (Table I). Of the total suspected patients, 13 were confirmed as leptospirosis by flaB PCR and 14 by rrs PCR, respectively,

TABLE II

Leptospira sequence identity related to disease complications

Specimen number (SLUSJ_)	Identity	Sequence similarity (%)	GenBank accession	Disease complication
1	*L. interrogans*	100	KP732501	Myocarditis
2	*L. interrogans*	100	KP732502	Acute renal failure
3	*L. interrogans* strain Canicola	100	KP732503	Acute renal failure
4	*L. interrogans* strain Canicola	100	KP732504	No complications
12	*L. borgpetersenni* strain *sejroe*	100	KP732506	Liver insufficiency
16	*L. interrogans*	100	KP732508	No complications
19	*L. interrogans* strain Canicola	100	KP732507	Liver insufficiency
23	*L. interrogans* strain	100	KP732509	Liver failure
70	*L. borgpetersenii* strain	99	KP732510	Liver failure
111	*L. interrogans*	99	KP732511	Myocarditis
119	*L. interrogans* strain Canicola	100	KP732512	Acute renal failure
160	*L. interrogans*	100	KP732513	Acute renal failure
176	*L. kirschneri* H2	100	KP732514	Acute renal failure
181	*L. interrogans*	99	KP732515	No complications

TABLE III

Comparison of selected features of leptospirosis outbreaks in Sri Lanka reported in 2008 and 2011 with the current study

Feature	2008[a]	2011[b]	2013[c]
Outbreak	Central province	North central province	Western and southern provinces
Period	Throughout the year	Following heavy rains and floods in first quarter of the year	Throughout the year
Predominant species	*Leptospira interrogans* (20/26)	*Leptospira kirschneri* (26/32)	*L. interrogans* (11/14)
Median duration of fever (IQR)	6 (4-8)	6 (2-8)	6 (4-8)
Renal failure (%)	13.8	21.9	35.7
Myocarditis (%)	10.3	15.6	14.3

a: Agampodi et al. (2011); *b*: Agampodi et al. (2014); *c*: current study; IQR: interquartile range.

according to the LERG guideline (WHO 2010). The rapid immunochromatographic assay (Leptocheck) used in this study had a sensitivity of 93% (Bandara et al. 2014) while the PCR was less sensitive. The high sensitivity of rapid immunochromatographic assay may have been associated with false positives. Similar observations were seen in a study done in India (Panwala et al. 2011). In this study the low PCR positivity may be explained by limited survival of the organism in the collected blood sample, immune system responses, prior use of antibiotics, DNA degradation during transportation and varied level of bacteraemia (Smythe et al. 2002).

RFLP has been used by several researchers to differentiate genotypes of *Leptospira* (Kawabata et al. 2001, Zakeri et al. 2010). The two restriction enzymes, Hae III and Hind III, used in our study were unable to differentiate between *L. interrogans* serovar Canicola and Pyrogenes. However, Hae 111 digestion was more discriminative than Hind 111 digestion for differentiating *L. interrogans* from *L. borgpetersenii*. Thus, its use in *Leptospira* genotyping is limited which is in line with studies done globally (Kawabata et al. 2001). Therefore, we used a more discriminative 16S rDNA sequencing method. Phylogenetic analysis of *Leptospira* indicates the presence of three clades namely, the pathogenic serovars, nonpathogenic serovars and intermediate group. While the rrs primer is able to identify both pathogenic and intermediate *Leptospira* species, flaB primers amplify only the pathogenic strains of *Leptospira* (Agampodi et al. 2011, Boonsilp et al. 2011, Natarajaseenivasan

et al. 2012). In the current study, SLUSJ_111 gave a positive PCR with rrs, but was negative with the flaB PCR. This can occur as a result of an intermediate strain or due to varying degree of sensitivity of the two assays. In the blast search of the amplified rrs sequence of SLUSJ_ 111 revealed an identity of 99% with *L. interrogans*. However, there is still a possibility of this being an intermediate strain because in the current study only a segment of *rrs* gene was subjected to sequencing. Intermediate species of *Leptospira* such as *Leptospira broomii, Leptospira inadai, Leptospira licerasiae, Leptospira wollfi* and *L. fainei* has been reported to cause acute febrile illness (Levett 2001). However there is no documented report of intermediate strains causing leptospirosis in Sri Lanka thus far.

In this study *L. interrogans* strains were the most common cause of disease followed by *L. borgpetersenii* and *L.kirschneri* strains. Circulating *L. interrogans* strains showed a 100% similarity to the 2008 strain which was isolated from central province in Sri Lanka (Agampodi et al. 2011). The strains isolated in this study showed 100% similarity to *L. interrogans* which was found to be the predominant strain in the current study and had been reported in Sri Lanka in 2008 outbreak. This strain was identified as a highly virulent strain (Agampodi et al. 2013). Moreover it has been reported from China and the Andaman Islands and seems to be associated with both severe and nonsevere disease (Agampodi et al. 2013).

Among 14 confirmed leptospirosis patients, only 11 developed complications whilst four were managed in intensive care units. Renal failure was the most common (45%) complication seen in the current study as seen in 2008 study (Agampodi et al. 2011) (Table III). Further in the current study, *L. interrogans* was the main cause of renal failure followed by hepatic insufficiency and myocarditis. *L. borgpetersenii* and *L. kirschneri* were not detected in the 2008 outbreak, but they have been reported previously during the 1960s and in the recent past from human and animal sources in Sri Lanka (Brenner et al. 1999, Koizumi et al. 2009, Agampodi et al. 2011, 2014). However, circulation of *L. borgpetersenii* among humans has not been well documented previously although it has been found among dairy cattle (Gamage et al. 2014). Cattle may be the source of infection in these two patients.

This study was conducted in the western and southern provinces of Sri Lanka having a different climatic, geographical and socioeconomical conditions when compared to the previous studies done in central and mid central provinces. This study highlights the evolutionary pattern of circulating strains in different time frames in Sri Lanka. In conclusion, *L. interrogans* was the predominant circulating strain in western and southern provinces in 2013 in Sri Lanka. The current data will contribute to determining molecular epidemiological diversity both in Sri Lanka and globally.

ACKNOWLEDGEMENTS

To the staff members at the Department of Microbiology, University of Sri Jayewardenepura, to the consultant physicians, to hospital staff members in the respective hospitals in Sri Lanka, to Dr Lilani Karunanayake, Head of Bacteriology division of Medical Research Institute, to Ms Rathnamali Perera, for providing diagnostic facility for MAT, and to Dr Menaka Hapugoda, Faculty of Medicine, University of Kelaniya, for providing serological diagnostic kits.

REFERENCES

Agampodi SB, Dahanayaka NJ, Bandaranayaka AK, Perera M, Priyankara S, Weerawansa P, Matthias MA, Vinetz JM 2014. Regional differences of leptospirosis in Sri Lanka: observations from a flood-associated outbreak in 2011. *PLoS Negl Trop Dis 8*: e2626.

Agampodi SB, Matthias MA, Moreno AC, Vinetz JM 2012. Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. *Clin Infect Dis 54*: 1249-1255.

Agampodi SB, Moreno AC, Vinetz JM, Matthias MA 2013. Utility and limitations of direct multi-*locus* sequence typing on qPCR-positive blood to determine infecting *Leptospira* strain. *Am J Trop Med Hyg 88*: 184-185.

Agampodi SB, Peacock SJ, Thevanesam V, Nugegoda DB, Smythe L, Thaipadungpanit J, Craig SB, Burns MA, Dohnt M, Boonsilp S, Senaratne T, Kumara A, Palihawadana P, Perera S, Vinetz JM 2011. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. *Am J Trop Med Hyg 85*: 471-478.

Bandara K, Gunasekara C, Weerasekara M, Ranasinghe N, Hapugoda M, Marasinghe C, Perera N, Gunapala A, Dickmadugoda N, Jayalath P, Siwagnanam FG, Fernando N 2014. Evaluation of three commercial rapid immunochromatographic kits for the presumptive identification of leptospirosis in Sri Lanka. Proceedings of the 1st International Conference on Multidisciplinary Approaches, University of Sri Jayewardenepura, August 13-14 2014 Sri Lanka, Faculty of Graduate Studies, University of Sri Jayewardenepura, Sri Lanka, p. 196.

Boonsilp S, Thaipadungpanit J, Amornchai P, Wuthiekanun V, Chierakul W, Limmathurotsakul D, Day NP, Peacock SJ 2011. Molecular detection and speciation of pathogenic *Leptospira* spp in blood from patients with culture-negative leptospirosis. *BMC Infect Dis 11*: 338.

Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS 1999. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new Leptospira genomospecies. *Int J Syst Bacteriol 49*: 839-858.

Gamage CD, Koizumi N, Perera AK, Muto M, Nwafor-Okoli C, Ranasinghe S, Kularatne SA, Rajapakse RP, Kanda K, Lee RB, Obayashi Y, Ohnishi M, Tamashiro H 2014. Carrier status of leptospirosis among cattle in Sri Lanka: a zoonotic threat to public health. *Transbound Emerg Dis 61*: 91-96.

Kawabata H, Dancel LA, Villanueva SY, Yanagihara Y, Koizumi N, Watanabe H 2001. flaB-polymerase chain reaction (flaB-PCR) and its restriction fragment length polymorphism (RFLP) analysis are an efficient tool for detection and identification of *Leptospira* spp. *Microbiol Immunol 45*: 491-496.

Koizumi N, Muto M, Tanikawa T, Mizutani H, Sohmura Y, Hayashi E, Akao N, Hoshino M, Kawabata H, Watanabe H 2009. Human leptospirosis cases and the prevalence of rats harbouring *Leptospira interrogans* in urban areas of Tokyo, Japan. *J Med Microbiol 58*: 1227-1230.

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

Natarajaseenivasan K, Raja V, Narayanan R 2012. Rapid diagnosis of leptospirosis in patients with different clinical manifestations by 16S rRNA gene based nested PCR. *Saudi J Biol Sci 19*: 151-155.

Nwafor-Okoli C, Koizumi N, Kularatne SA, Rajapakse J, Gamage CD, Muto M, Suzuki M, Lee RB, Kanda K, Obayashi Y, Tamashiro H

2012. Leptospira infection at the University of Peradeniya Teaching Hospital, Sri Lanka: clinical and laboratory investigations. *Southeast Asian J Trop Med Public Health 43*: 943-950.

Panwala T, Mulla S, Patel P 2011. Seroprevalece of leptospirosis in South Gujarat region by evaluating the two rapid commercial diagnostic kits against the MAT test for detection of antibodies to *Leptospira interrogans*. *National Journal of Community Medicine 2*: 64-70.

Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ, McKay DB 2002. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infect Dis 2*: 13.

Victoriano AF, Smythe LD, Gloriani-Barzaga N, Cavinta LL, Kasai T, Limpakarnjanarat K, Ong BL, Gongal G, Hall J, Coulombe CA, Yanagihara Y, Yoshida S, Adler B 2009. Leptospirosis in the Asia Pacific Region. *BMC Infect Dis 9*: 147.

WHO - World Health Organization 2010. *Report of the first meeting of the leptospirosis burden epidemiology reference group*, WHO, Geneva, 40 pp.

Zakeri S, Sepahian N, Afsharpad M, Esfandiari B, Ziapour P, Djadid ND 2010. Molecular epidemiology of leptospirosis in northern Iran by nested polymerase chain reaction/restriction fragment length polymorphism and sequencing methods. *Am J Trop Med Hyg 82*: 899-903.

Case report :

Leptospirosis- A Physician's dilemma or diagnostic enigma?

Dr. Mohit Bhatia[1] , Dr. Umapathy B.L[2]

[1] Senior Resident, Department of Microbiology, Govind Ballabh Pant Institute of Post Graduate Medical Education and Research, Jawahar Lal Nehru Marg, New Delhi-110002.

[2] Professor, Department of Microbiology, Sree Mookambika Institute of Medical Sciences, Kulasekharam, Kanyakumari District, Tamil Nadu (India) -629161

Corresponding author: Dr. Mohit Bhatia

Abstract:

A two years old female child was admitted in a tertiary care hospital in June 2012 with history of high grade fever of two weeks duration. On examination, no systemic abnormality was detected. Acute and convalescent sera of this patient were positive by widal test and rapid leptospira serological tests like Macroscopic Slide Agglutination Test (MSAT), IgM Enzyme Linked Immunosorbent Assay (IgM ELISA) and immunochromatographic card test (IgM Leptocheck). However, both of these serum samples were negative by Microsopic Agglutination Test (MAT). Blood culture was sterile. Leptospires were isolated from urine sample of this patient and identified as *Leptospira inadai* by Polymerase Chain Reaction (PCR). This patient was treated successfully with Amoxicillin/Clavulanic acid syrup and discharged after one week of admission.

Key-words: *Leptospira inadai,* widal, MSAT, IgM ELISA, IgM Leptocheck, MAT, PCR

Introduction:

Leptospirosis is an emerging infectious disease which is often missed clinically. [1] The signs & symptoms of leptospirosis resemble a wide range of infectious diseases.[2] A high index of suspicion is needed in endemic areas & leptospirosis must be considered when a patient presents with acute onset of fever, headache & myalgia. The diagnosis of leptospirosis in humans is almost entirely dependent on laboratory findings. The most frequently used diagnostic approach for leptospirosis has been that of serology.[3,4] We hereby present a case of human infection caused by a rare species *Leptospira inadai*.

Case History:

A two years old female child was admitted in a tertiary care hospital during monsoon season in June, 2012, with history of high grade, intermittent fever of insidious onset of two weeks duration along with headache and myalgia. The patient belonged to an economically backward family living under poor sanitary conditions. Further interrogation revealed the presence of numerous rats in their house, with many open drains around their residence and history of barefoot walking. Many similar cases of febrile illness had been reported in their locality during that season. However, they were unaware of the diagnosis of those cases. On examination, the patient was febrile (Temperature-101.5˚F). No systemic abnormality was detected. Laboratory investigations showed

(1) Hemoglobin: 9.3g/dl
(2) Total Leucocyte Count: 9700/Cu.mm of blood
(3) Differential Leucocyte Count: Polymorphs-34%; Lymphocytes-61%; Monocytes-5%
(4) Platelet count: 2.3 lakhs/Cu.mm of blood

(5) Peripheral smear for Malaria parasite: Negative

(6) Urine Routine and Microscopy: No abnormality detected

(7) Blood culture : Sterile

(8) Widal test: Two serum samples of this patient were collected one week apart and labeled as acute and convalescent respectively. The results obtained were as follows: 'STO' 1:80, 'STH':1:320, 'SPAH' < 1:20 & 'SPBH' <1:20 (acute sample) and 'STO' 1:160, 'STH' 1:1280, 'SPAH' <1:20 & 'SPBH' < 1:20 (convalescent sample).

As per the requisition received from pediatrician, blood and urine samples of this patient were also evaluated for leptospirosis as per standard procedures:[5]

(1) Dark Field Microscopy (DFM) of blood and urine samples: Negative

(2) Blood culture for leptospirosis using commercially available Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid medium (BD-Difco): Sterile

(3) Urine culture for leptospirosis using EMJH medium containing 100µg/ml 5-Fluoro Uracil (Rolex Chemical Industries) as selective agent: Leptospires were grown after 96 hours of incubation as indicated by Dinger's ring (ring of growth present on sub-surface) and confirmed by DFM. (**Fig 1)**

The following rapid leptospira serological tests were also performed on acute and convalescent sera of this patient: Macroscopic Slide Agglutination Test (MSAT; Bio-Rad), immunochromatographic card test (IgM Leptocheck; Zephyr Biomedicals), IgM Enzyme Linked Immuno Sorbent Assay (IgM ELISA; J. Mitra & Co. Pvt. Ltd.). Both serum samples were positive by all the aforementioned tests.

Since the acute and convalescent sera of this patient were tested positive by widal and all aforementioned leptospira serological tests, the Microscopic Agglutination Test (MAT) was performed on these sera upon receiving a special requisition from the department of Pediatrics. MAT was performed at Regional Medical Research Centre (Indian Council of Medical Research), WHO collaborating centre for diagnosis, reference, research & training in leptospirosis, Port Blair, Andaman and Nicobar islands (India) using the following serovars: *Australis, Bankinang, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Pomona, Pyrogenes* & *Hardjo*. Both serum samples were negative by MAT.

The urine culture leptospira isolate was sent to Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), Bengaluru for confirmation by PCR. This isolate was characterized at species level as Leptospira inadai by using partial RNA polymerase β-subunit (rpoB) gene sequences.

Based on these results, this patient was treated with Amoxicillin/Clavulanic acid syrup (125/31.25 mg) 2.5 ml thrice a day for seven days. The patient recovered and was discharged after one week of admission.

Discussion:

Leptospirosis is considered as the most common zoonotic infection in the world with higher incidence in the tropics than temperate regions.[6] Though it is sub-clinical or mild in most cases, severe illness can sometimes end fatally.[1] The clinical presentation is difficult to distinguish from dengue, malaria,

www.ijbamr.com P ISSN: 2250-284X , E ISSN : 2250-2858

influenza & many other diseases characterized by fever, headache & myalgia. The differential diagnosis of leptospirosis depends on the epidemiology of acute febrile illnesses in the particular area. The mainstay of diagnosis is microbiological which has various shortcomings.

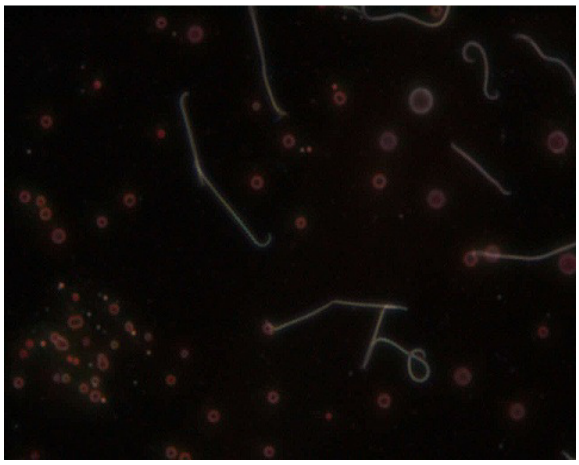
Both blood and urine samples of this patient were tested negative by DFM which has not been accepted for diagnostic purposes as it is considered insensitive and the results are non specific.[1] IgM Leptocheck, MSAT and Leptospira IgM ELISA are rapid sensitive serological diagnostic tests for leptospirosis. Many studies have shown that these tests have very high Positive Predictive Values (PPV).[4,7,8] Widal test was positive on paired sera of this patient with rising titres of both 'STO' and 'STH' antigens. This may be inferred as co-infection or serological cross-reactivity. Dual infections with leptospires & other etiologic agents like Dengue virus, Human Immunodeficiency Virus, Hepatitis B & E viruses etc. have rarely been reported. Serological cross-reactivity between leptospirosis and other infectious diseases has also been reported.[1,3,4]

However, both serum samples were negative by MAT which is considered as serological gold standard for the diagnosis of leptospirosis.[9] It is imperative to know the circulating *Leptospira* species/serovars in animals and humans in different geographical locations in order to investigate the prevalence of *Leptospira* species during monitoring of the leptospirosis. This helps in appropriate use of panel of leptospira serovars in the MAT for providing proper diagnosis without false negative results.[10] Hence, due to non-inclusion of *Leptospira inadai* in the panel of serovars used for MAT, negative result was obtained.

Leptospira inadai was isolated from urine sample of this patient. Phylogenetic analysis based on 16S rRNA gene sequences have identified three clades of *Leptospira spp.* containing branches that, with few exceptions, reflect species designations based on the pathogenicity status (pathogenic, saprophytic & intermediate strains of unclear pathogenicity) and *Leptospira inadai* belongs to intermediate branch of unclear pathogenicity.[11] In India, earlier sporadic human case reports with *Leptospira inadai* infection and circulation of *Leptospira inadai* in reservoir hosts have been reported.[12,13]

Given the clinico-epidemiological background and results of various laboratory tests, this patient was successfully treated with amoxicillin/clavulanic acid and discharged subsequently. This case report affirms the long held belief that diagnosis of leptospirosis (both laboratory & clinical) is an uphill task. It is an enigmatic disease which presents with various challenges for both clinicians & laboratory physicians.

Fig 1: Leptospires under Dark Field Microscope (x1000)



www.ijbamr.com P ISSN: 2250-284X , E ISSN : 2250-2858

Acknowledgements:

Dr. Pushpalatha K, Professor & HOD, Department of Pediatrics, ESIC-MC & PGIMSR, Rajajinagar, Bengaluru, Dr.P.Vijayachari, Director, Regional Medical Research Centre, Port Blair, Andaman & Nicobar Islands, India, Dr. Vimal Raj, Regional Medical Research Centre, Port Blair, Andaman & Nicobar Islands, India, Dr.K. Prabhudas, Dr.H.Rahman, Dr.M.R.Gajendragad, Dr.V.Balamurugan, Dr.Nagalingam, Mrs.Sushma and K Assadi, (Ex-Director, Director, Principal Scientist, Senior Scientist, Scientist and Trainee respectively at Leptospira Lab, PD_ADMAS Bengaluru

References:

1. Prasad SR, Rajini M. Leptospirosis: An Overview. The Journal of the Academy of Clinical Microbiologists 2008;10(2):89-97

2.Sharma KK, Kalawat U. Early diagnosis of leptospirosis by conventional methods: One year prospective study. Indian J Pathol Microbiol 2008; 5:209-11

3. Mary DB, David AA, Sandra LB, Christopher WW, Tin A, Richard AS et al. Evaluation of Four Commercially Available Rapid Serological Tests for Diagnosis of Leptospirosis. J Clin Microbiol 2003 Feb;41(2):803-09

4. Pimjai N, Piyada W, Wimol P, Ornnalin L, Paijit W. A Comparitive Evaluation of Different Methods for the Serological Diagnosis of Leptospirosis. J Trop Med Parasitol 2000 December;23(2):59-65

5. Vijayachari P, Sameer Sharma & Natarajaseenivasan K. Laboratory Diagnosis. In: Leptospirosis Laboratory Manual. Regional Medical Research centre, Port Blair ,WHO Country office for India: WHO;2007.p.27-45

6. Vijayachari P. Introduction. In: Leptospirosis Laboratory Manual. Regional Medical Research centre, Port Blair, WHO Country office for India:WHO;2007. p.1-3

7. Brandao AP, Camargo ED, Silva ED, Silva MV, Abrao RV. Macroscopic Agglutination Test for rapid diagnosis of human Leptospirosis. J Clin Microbiol 1998 Nov;36(11):3138-42

8. Tanvi P, Summaiya M, Parul P. Seroprevalance of leptospirosis in south Gujarat region by evaluating the two rapid commercial diagnostic kits against the MAT test for detection of antibodies to Leptospira interrogans. National Journal of Community Medicine 2011;2(1):64-70

9. Sambasiva RR, Naveen G, Bhalla P, Agarwal SK. Leptospirosis in India and the rest of the world. Braz J Infect Dis 2003;7(3):178-93

10. Balamurugan et al.: Characterization of leptospira isolates from animals and humans: phylogenetic analysis identifies the prevalence of intermediate species in India. Springer Plus 2013; 2:362

11. Gustavo MC, Mathieu P. A century of Leptospira strain typing. Infection, Genetics and Evolution 2009; 9:760-68

12. Gangadhar NL, Rajasekhar M, Smythe LD, Norris MA, Symonds ML, Dohnt MF. Reservoir hosts of *Leptospira* inadai in India. Rev Sci Tech OIE 2000;19(3):793-799

13. Gangadhar NL, Prabhudas K, Bhushan S, Sulthana M, Barbuddhe SB, Rhaman H. *Leptospira* infection in animals and humans: a potential public health risk in India. Rev Sci Tech OIE 2008; 27(3):885-892

Abstract 17

Assessment of oxidative stress in severe leptopsirosis patients

Wickramasinghe SA[1], Eugene EJ[1], De Silva HJ[2], Rajapakse S[3], Handunnetti SM[1]

[1]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo
[2]Department of Medicine, Faculty of Medicine, University of Kelaniya
[3]Department of Clinical Medicine, Faculty of Medicine, University of Colombo

Release of reactive oxygen and reactive nitrogen species (ROS and RNS) contribute to increased oxidative stress and to tissue damage which is thought to lead to multi-organ failure in Leptospirosis. The antioxidant capacity of serum provides a measure of overall protection against oxidative damage. Level of oxidative stress caused by ROS and RNS (ie., nitric oxide (NO▪) was assessed in leptospirosis patients in the present study. Of 81 clinically suspected leptospirosis patients recruited, 40 were confirmed by Leptocheck WB test (LCT). They were clinically categorized as severe (n=24) and mild (n=16) cases. LCT negative patients were considered as non-leptospirosis fever (NLF) controls (n=41) and a healthy control group (n=20) was also included. NO▪ was measured by determining serum NO_2- and NOx (NO_2-+NO_3-) levels using direct Griess and modified Griess assays respectively. To assess the damage caused by ROS, serum anti oxidant capacity (AOC) was measured using ABTS decolorization assay and results were expressed as Trolox equivalent (TE) µM/mg proteins. Severe leptospirosis patients had significantly high serum NO2-levels compared to NLF and healthy controls (1.8±0.11µM, 1.2±0.08µM and 1.1±0.08µM respectively; P<0.001). Serum NOx levels (18.5±1.50µM) of severe patients were significantly higher compared to mild leptospirosis patients, NLF and healthy control groups (14.3±0.94µM, 12.3±0.89µM and 5.5±0.27µM; P<0.05 respectively. Both severe and mild leptospriosis patients had comparable AOC levels (1.05±0.04 and 1.03±0.09 µM/mg protein respectively) but the levels were significantly higher compared to NLF and healthy control groups (0.97±0.04 and 0.92±0.03 µM/mg protein; P<0.05 respectively). Thus, serum NOx could be used as a prognostic indicator of severity of leptospriosis.

This work was supported by the IBMBB and constitutes part of the MSc studies of SAW.

‹ Abstract 16 up Abstract 18 ›

ABSTRACT BOOK (2012)

INSTITUTE LECTURE
ORAL COMMUNICATIONS
POSTER PRESENTATIONS
Abstract 14
Abstract 15
Abstract 16
Abstract 17
Abstract 18
Abstract 19
Abstract 20
Abstract 21
Abstract 22
Abstract 23
Abstract 24
Abstract 25
Abstract 26
Abstract 27
Abstract 28
Abstract 29
UPDATES I-IV

Institute of Biochemistry, Molecular Biology and Biotechnology
90, Cumaratunga Munidasa Mawatha,
Colombo 03
Sri Lanka

Telephone : +94 11 2552528
Fax : +94 11 2553683
E-mail: info@ibmbb.cmb.ac.lk

Rajapakse et al. Annals of Clinical Microbiology and Antimicrobials (2015) 14:2
DOI 10.1186/s12941-014-0060-2

ANNALS OF CLINICAL MICROBIOLOGY AND ANTIMICROBIALS

REVIEW Open Access

Current immunological and molecular tools for leptospirosis: diagnostics, vaccine design, and biomarkers for predicting severity

Senaka Rajapakse[1*], Chaturaka Rodrigo[1], Shiroma M Handunnetti[2] and Sumadhya Deepika Fernando[3]

Abstract

Leptospirosis is a zoonotic spirochaetal illness that is endemic in many tropical countries. The research base on leptospirosis is not as strong as other tropical infections such as malaria. However, it is a lethal infection that can attack many vital organs in its severe form, leading to multi-organ dysfunction syndrome and death. There are many gaps in knowledge regarding the pathophysiology of leptospirosis and the role of host immunity in causing symptoms. This hinders essential steps in combating disease, such as developing a potential vaccine. Another major problem with leptospirosis is the lack of an easy to perform, accurate diagnostic tests. Many clinicians in resource limited settings resort to clinical judgment in diagnosing leptospirosis. This is unfortunate, as many other diseases such as dengue, hanta virus, rickettsial infections, and even severe bacterial sepsis, can mimic leptospirosis. Another interesting problem is the prediction of disease severity at the onset of the illness. The majority of patients recover from leptospirosis with only a mild febrile illness, while a few others have severe illness with multi-organ failure. Clinical features are poor predictors of potential severity of infection, and therefore the search is on for potential biomarkers that can serve as early warnings for severe disease. This review concentrates on these three important aspects of this neglected tropical disease: diagnostics, developing a vaccine, and potential biomarkers to predict disease severity.

Keywords: Leptospirosis, Vaccine, Biomarkers, Diagnosis

Introduction

Leptospirosis is a zoonotic disease caused by spirochaetes of the genus *Leptospira*. The disease results in high morbidity and considerable mortality in areas of high prevalence [1]. It is estimated that around 10,000 cases of severe leptospirosis are hospitalized annually worldwide [2]. The disease is endemic in areas with high rainfall, close human contact with livestock, poor sanitation and workplace exposure to the organism [3]. There are currently 14 identified potentially pathogenic species of leptospira (9 definite and 5 intermediate). Any mammal has the potential to be the reservoir for the organism, but it is predominantly rodents who play a role in transmitting infection to humans. The organisms can be transferred to humans through contact with body fluids and urine of infected animals, with entry of the organisms occurring through mucosal surfaces or breached skin [2].

Primarily manifesting as an acute febrile illness, severe forms of leptospirosis affect multiple organ systems, resulting in acute kidney injury, pulmonary haemorrhage, hepatitis, myocarditis, disseminated intravascular coagulation, and meningo-encephalitis. The case fatality rate in severe leptospirosis can exceed 40% [4]. It is postulated that severe disease is driven largely by the host immunological response rather than the pathogen's virulence. There are a multitude of unresolved, practically relevant areas on this illness that need to be addressed by further research. In this review, we focus on three important areas, i.e., diagnostics, vaccine development, and identification of biomarkers of disease severity.

Methods

A MEDLINE search was performed for articles with the keywords 'leptospirosis' OR 'leptospira' OR 'Weil's' OR

* Correspondence: senaka@med.cmb.ac.lk
[1]Department of Clinical Medicine, Faculty of Medicine, University of Colombo, 25, Kynsey Road, Colombo 08, Sri Lanka
Full list of author information is available at the end of the article

'Weil' in title or abstract. The search was restricted to articles published in English within the last 15 years (1998 – September 2013) as they would contain more recent data. The search was also restricted to studies reporting on humans (not animals). There were 1442 abstracts in the original search with these restrictions. The software Endnote X3 was used to filter articles. Bibliographies of cited literature were also searched. All abstracts were read through independently by the three authors, and relevant papers were identified for review of the full papers. Related papers were also included. We reviewed 159 selected full papers.

These sources were screened for a well described methodology, accurate statistical analysis and an adequate sample size where relevant. Data sources included reviews published in core clinical journals, cohort studies, interventional studies, case control studies, cross sectional analysis and epidemiological data. Suitable data was available in 49 papers from the initially selected 159.

Leptospirosis: diagnostic issues

Laboratory diagnosis of leptospirosis remains a challenge. There are many diagnostic tests for leptospirosis. These can be broadly divided according to their methodology into: a) methods demonstrating the organism in culture or clinical specimens, b) immunological methods, and c) genomic methods.

Direct demonstration of organisms

The simplest diagnostic procedure is demonstrating the organism in urine or blood with dark ground microscopy (DGM). However, the sensitivity and specificity is questionable, despite the low cost. In addition, ideal diagnostic conditions with DGM require the specimen to be prepared from culture, which is very difficult to achieve since the organism is fastidious. Chandrasekaran et al. [5] compared the usefulness of DGM vs. IgM ELISA, and concluded that DGM had high positivity in patients with clinically suspected leptospirosis compared to ELISA (95.5% vs. 64.7%). The positivity of DGM diminished, and that of ELISA increased (though still DGM had higher positivity) with the duration of infection. Comparison was not made against a gold standard in this case, and the study simply compared positivity with the two tests in clinically suspected cases. In another study of 297 samples, sensitivity and specificity of DGM was around 60% [6].

Immunological diagnostics

The immunological reference standard for diagnosis of leptospirosis is the microscopic agglutination test (MAT). However, this test involves the cumbersome procedure of reacting the patient's serum with different panels of live leptospira antigens. MAT is not specific for IgM, and detects both IgM and IgG, and may not be able to differentiate acute from previous infection. Furthermore, there is little evidence on how long IgG antibodies persist in blood after acute infection. Thus ideally, the test requires two samples (acute and convalescent) for confirmation. In a clinical setting where rapid decision making is necessary, MAT is not be the ideal test to go by for diagnostic confirmation.

Other immunological tests available include IgM ELISA, microcapsule agglutination test, Lepto-dipstick, Lepto Dri Dot, and Leptocheck-WB test. These allow rapid diagnosis, and are simpler to perform than MAT. Still, all these tests can be negative in early leptospirosis as it takes time for antibodies to form. The sensitivities and specificities of the tests vary depending on the antibodies present and the leptospira antigen used. For example Boonyod and colleagues [7] demonstrated that a rapid diagnostic test using a dipstick for the outer membrane protein (OMP) LipL32, which is expressed in pathogenic leptospira, had good sensitivity and specificity to MAT (100% and 98.3% respectively). The suitability of this antigen for pathogenic leptospira diagnosis has been independently confirmed by others [8-10]. A group of investigators in Brazil assessed the use of *Leptospira* immunoglobulin (Lig)-like proteins as antigens to react with IgM antibodies in patient's sera in an immunoblot assay. This had a sensitivity of 81% during the first 7 days of illness. Neves and colleagues [11] identified two proteins, namely Lp29 and Lp49, which were reactive with sera of patients during an outbreak in Brazil. These proteins were identified after screening the *L. interrogans* genome for potential sequences that code for outer membrane proteins. The IgM for these proteins were detected in sera of patients in both acute and convalescent phases, and IgM against Lp29 was detected when the MAT was negative in the acute phase of illness. However, it was not confirmed whether these proteins were present in all pathogenic serovars of leptospirosis. An IgM immunoblot test against antigens of several leptospira serovars prevalent in Thailand yielded positive results, with a sensitivity of 88% during the first three days since onset of symptoms (corresponding MAT sensitivity in this early sample was just 2%). ELISA assays based on recombinant products of OMPs are developed for locally circulating virulent organisms. Whether they would be useful outside a particular geographical area is doubtful. In a large scale study in Andaman Islands, researchers developed an IgM ELISA study for two OMPs (OmpL1 and LipL41) of locally prevalent virulent leptospira serovars that caused severe pulmonary leptospirosis [12]. The test had sensitivities and specificities ranging between 80-90% compared against MAT but may not be universally applicable for different serovars that are prevalent in other areas. Senthilkumar et al. [13], in an attempt to develop a rapid diagnostic method, assessed recombinant LipL41 protein as an antigen to be used in latex agglutination test (LAT)

and flow through assays. The protein was conserved among all pathogenic species of leptospirosis. Both tests took less than 5 minutes to complete and had good sensitivities and specificities when compared against MAT (sensitivity and specificity 89.7% and 90.4% respectively for LAT, 77% and 89% for flow through assay). Other recombinant conserved proteins of pathogenic leptospira that have yielded good results in immunological diagnostics include: rligA [14], Hap1/lipL32 [15], and rLoa22 [16].

Another interesting aspect to leptospirosis diagnosis by immunological methods was adopted by Lin et al. [17]. They considered five immunodominant epitopes of three OMPs of pathogenic leptospira (OmpL1, LipL21, and LipL32) and constructed a synthetic gene (rlmp). The purified protein product of this gene was used as an antigen to react with patient sera (with both IgM and IgG antibodies) of confirmed leptospirosis patients in an ELISA test. The results were encouraging with no cross reactions and false positives in control groups, and detecting all MAT positive leptospirosis with the new test. In a similar experiment, Sun et al. [18] created a recombinant fusion protein of the same antigens that was later used in an IgM ELISA for early diagnosis of leptospirosis. The ELISA with the recombinant protein yielded better results (>95% sensitivity in a sample of 493 leptospirosis patients) than ELISA tests using each of the individual antigens. Additionally they demonstrated that this antigen did not cross react with sera of patients with non-leptospirosis fevers, such as dengue and typhus.

Finally, a recently published meta-analysis of ELISA diagnostic tests for leptospirosis holds that they have a fairly good sensitivity and specificity (77% and 91% respectively; area under the curve 0.964). The drawbacks were the heterogeneity among the tests and the lower yield in the initial phase of the illness [19]. This remains the main problem with IgM ELISA tests for leptospirosis, i.e., heterogeneity between different antigens used for testing essentially affects sensitivity among different strains of the organism.

While being less sophisticated and time consuming than the MAT, ELISA tests also need considerable laboratory support. Rapid diagnostic tests (RDT) are an alternative for on-field diagnosis with minimal laboratory support. Goris and colleagues [20] evaluated three commercially available RDTs (LeptoTek Dri Dot, LeptoTek Lateral Flow, and Leptocheck-WB) against the MAT and ELISA test results for the same samples. All three tests had sensitivity of more than 75% and specificity of at least 95%. However in order to obtain a better sensitivity, at least two samples had to be tested per patient (sensitivity for single samples ranged from 51-69%). It was concluded that RDTs alone cannot be relied upon to diagnose leptospirosis, especially in the earlier stages of the illness.

However, all these comparisons of different immunological diagnostic tests need a gold standard for a valid comparison. Until recently this gold standard was MAT (or culture which has low sensitivity as the organism is fastidious). Unfortunately, the MAT is in itself an imperfect gold standard, which makes the sensitivities and specificities of other tests judged against it less reliable and hence has to be interpreted with caution (see below) [21].

Genomic diagnostics

Genomic diagnostic tests have the advantage of being positive early in disease, but have the disadvantages of limited availability and high cost. There are several diagnostic techniques that can be employed in the genomic diagnosis. These are outlined below.

Polymerase chain reaction (PCR): Involves amplifying DNA sequences specific to the organism, using primers. Provided the sequence amplified is specific to the pathogen, this method has the potential to be 100% specific. Gravekamp et al. [22] developed two groups of primers (G1 & G2 and B64 I & B64 II) that were capable of diagnosing all genospecies of leptospira known upto the year 2003, and these had been used heavily in studies that required specific diagnosis of leptospirosis. De Abreu Fonseca et al. [23] compared the sensitivity and specificity of PCR against that of MAT and IgM ELISA in 124 serum samples (60 with confirmed leptospirosis). The specificity of PCR was 100% but the sensitivity varied between 44-62% with less sensitivity for samples collected later on in the infection. The sensitivities for MAT ranged between 69 -95% and increased with duration since infection (specificity of MAT ranged between 90 – 100%). Combination of PCR and ELISA increased the sensitivity to 93-95% during first week of infection. Similar findings have been demonstrated by other authors as well [24].

Arbitrarily primed PCR: This technique uses an arbitrary primer to amplify segments of DNA which on gel electrophoresis should produce a specific pattern of bands that is species specific. However, even within the same species, researchers have shown that arbitrary primed DNA banding patterns can differ.

Nucleic acid probes: This is a very specific technique that allows diagnosis of infection at a very early stage. Provided the probe is a specific one, it will enable species differentiation.

Restriction enzyme analysis (REA): Cleaving purified dsDNA of leptospira by restriction enzymes gives a specific DNA fingerprint when run on gel electrophoresis. Recognizing this pattern will enable to identify members of same species with same restriction sites. While this can be used as a diagnostic technique, application of this has also enabled to further genetically classify subspecies or identify new species that were previously thought to be a single species.

Random amplified polymorphic DNA fingerprinting (RAPD): This involves combination of arbitrary primer use and PCR to generate a unique pattern of genomic bands that is specific at species level. This technique has enabled rapid differentiation between different species but has the disadvantage of needing pure cultures to extract DNA.

Pulsed field gel electrophoresis: This is a technically cumbersome procedure of generating larger genomic fragments by restriction enzymes that need to be moved and separated by a special gel electrophoresis. While being a difficult process, it allows a relatively reproducible fractionation of an entire bacterial genome on a single gel.

Ribotyping: Ribosomal RNA (rRNA) is relatively well conserved within the species. Bacteriologists use probes on rRNA to identify the phylogenetic position of bacteria. It has been suggested that this tool may be useful in identifying the epidemiology and species differentiation of leptospira. Taking MAT/culture as the gold standard, Thaipadungpanit et al. [25] compared the diagnostic specificity and sensitivity of detection of genomic 16 s rRNA and lipL32 gene in 133 cases of leptospirosis (plus 133 controls). The diagnostic sensitivity was low with both tests, but was better in the 16 s rRNA assay (53 vs. 46%); specificity was high, but lower with 16 s rRNA (90 vs. 93%). The advantage of these tests compared to MAT is that detection of genomic material can be done at a very early stage of the illness without having to wait for antibody development. In Sri Lanka, Agampodi et al. [26] used quantitative PCR to amplify 16 s rRNA, and found that sensitivity was much better when serum was used as the source than whole blood (51 vs 18%). Quantitative leptospiraemia correlated with myocarditis, renal failure and multi organ failure. Furthermore, sensitivity of PCR was not affected by the duration of illness.

DNA sequencing: Sequencing nucleic acids at a particular genetic locus allows to identify interspecies differences and genetically classify different serovars. This is a laborious and expensive technique.

In an overall analysis of diagnostic tests for leptospirosis, the trend now is to find a test that will yield good results as early as possible in the disease process. Culture and MAT, though considered to be the gold standard, are clearly unsuitable in this regard as they are cumbersome and time consuming. Of the serological tests, several studies have indicated the possibility of utilizing antibodies against OMPs of pathogenic leptospira species for early diagnosis with good sensitivity and specificity. Most of these tests utilize IgM antibodies though some utilize IgG antibodies. However, the disadvantage is that the antigen against which the antibodies are developed may not be conserved among all pathogenic leptospirosis serovars. If that is the case then tests developed for circulating serovars in one locality may not be applicable to others. However, many proteins that have been mentioned above seem to be conserved across the pathogenic species. MAT, despite being the "gold standard" has its own problems. It requires the continuous maintenance of live leptospira antigens in a panel of different serovars. If the standard panel does not contain a locally prevalent serovar, again the diagnosis may be missed.

In most previous studies of new diagnostic tests, comparison has been made with MAT as the reference standard. The validity of comparing new immunodiagnostics with MAT as the "gold standard" has been debated [21], for the reasons mentioned earlier. Bayesian latent class modeling has been suggested over traditional gold standard analysis when evaluating new immunological diagnostic tests. The role of genetic testing has come to the fore recently mainly because of better sensitivity in early disease compared to MAT. Theoretically, genomic antigen detection would allow better and faster diagnosis, but these methods are not widely available, and are likely to be costly.

Developing a vaccine for leptospirosis

There is currently no widely used vaccine for leptospirosis. The first vaccine introduced for leptospirosis was a killed whole cell vaccine that consisted of formalin-killed leptospires [27]. Various studies report the duration of efficacy of whole cell vaccines to be between 6 months to 7 years. However, in most studies, the duration of protection was at best 3 years [27]. The problem with this vaccine is that its serovar specific [27]. The monovalent vaccine did not protect against infection by other serovars and therefore its protection is dependent on the locally isolated serovars. This fact, plus its side effects, has led to other options being explored in vaccine designing.

Leptospiral lipopolysacharides (LPS) are an area of interest for vaccine developers. However, immunity generated by these antigens was also considered to be serovar specific. Some success with LPS vaccines has been demonstrated in animal models.

Protein antigens are a mainstay of the current drive to develop a leptospirosis vaccine. The discovery of outer membrane proteins of leptospira that were common or conserved in pathogenic species has generated interest among immunologists and vaccinologists in developing a polyvalent vaccine that is effective against different pathogenic species with minimum side effects. Subunit vaccines cause less side effects than whole cell vaccines. The proteins of interest are: Omp L1 (transmembrane protein), LipL41 (outer membrane protein), LipL32/Hap-1, Leptospiral immunoglobulin-like proteins [28] and LemA [29]. Seixas and colleagues [30] evaluated the potential of using LipL32 with various vaccine platforms to induce immunity in an animal model (rBCG vaccine, DNA vaccine and a subunit vaccine). The protein was immunogenic and the subunit vaccine gave the highest

antibody titres. They further demonstrated that anti LipL32 inhibited leptospira growth in vitro.

The leptospiral immunoglobulin-like proteins consist of three proteins LigA, LigB and LigC. LigA and B have been to shown to have immunogenic potential in animal models [31]. LigB is universally present in all pathogenic leptospira serovars and therefore carries the best hope for being an immunogenic component in a recombinant universal leptospirosis vaccine [32]. Cao and colleagues [33] developed a fusion recombinant protein of two immunogenic proteins (immunoglobulin like proteins and LipL32) and the combined product (in various combinations) had good protective efficacy in a hamster model. The authors also noted that using LipL32 alone was not as successful as using the combined protein. However in a subsequent paper, a different group of investigators reported that when LipL32 is combined with B subunit of *E. coli* heat liable enterotoxin, it evoked a significant immunoprotective effect [34].

With sequencing of entire genomes of some pathogenic leptospira species, the possibility of isolating sequences that might code for membrane proteins that are potential candidates for vaccine development has opened up. Such areas can be recognized by scanning the entire genome with computer generated algorithms; identified genes can be cloned and their proteins purified to check for antigenicity [35-37]. This is a very complex process, but has immense potential for future vaccine development. Such putative protein products have been purified and tried in animal models with some promising results.

Overall, despite the advances in biotechnology, the only usable efficacious vaccine for leptospirosis to date are the whole cell inactivated vaccines. Vaccines based on recombinant membrane proteins have only been tried out in animals with limited success. The disadvantage of whole cell vaccines is that they are serovar specific (polyvalent vaccines can be made by using several serovars in one vaccine) and therefore can be used in a geographically restricted area. Nonetheless, given the limited progression on developing a universally useful vaccine active against all pathogenic serovars, the most cost effective measure for a developing country is to work on a locally effective killed whole cell vaccine.

Molecular markers of severe leptospirosis

Leptospirosis is a disease with a wide spectrum of manifestations. Only a minority of infected people will develop severe disease with multi-organ failure. This severe disease is seen with certain serovars but not all individuals infected with a particular serovar will develop severe disease. To make matters complex, the classification of serovars is a cumbersome process that is dependent on detailed immunological phenotyping. It does not relate with leptospira species categorization which is based on DNA analysis. While serovar diagnosis is relatively freely available, DNA based species categorization is only available in reference genetic laboratories. This creates a barrier in correlating clinical features with the infecting species.

Why certain infections in some people lead to severe disease, while others have a mild illness, is an unresolved mystery. Current thinking is that both pathogen related (infecting serovar/species, innoculum size) and host factors (immunological response) contribute to this heterogeneity. However, if certain markers of severe disease can be identified either in the host or the pathogen, it will be of great help in predicting severe disease.

One particular compound that has been of interest in this regard is nitric oxide. It is known that in a state of inflammation, release of inflammatory cytokines (TNF-α, IL-1,6) activate inducible nitric oxide synthatase (iNOS) to produce NO which is bacteriostatic. NO is metabolized to nitrite, which has a short half-life in blood, and then to nitrate. Estimation of NO activity can be made by measuring nitrites, nitrates or both. There is limited evidence as to which metabolite most accurately reflects NO activity in response to severe infection. Nitrite is likely to be more specific, as it has a short half life, and is less affected by renal function. It was hypothesized that in severe leptospirosis the level of NO may be elevated. This hypothesis was confirmed by two separate studies six years apart, where serum NO levels were shown to be raised in patients with symptomatic leptospirosis [38,39]. However, a paradox in serum nitrate concentrations (a surrogate marker of NO) has been demonstrated in malaria where people with severe malaria actually had paradoxically low total NOx (nitrite and nitrate) levels when it was corrected for serum creatinine. In severe leptospirosis, since there is renal impairment, it is possible that the raised NO level may not reflect increased synthetic activity but reduced clearance of NO via the kidneys. In fact a recently published paper by Kalugalage et al. [40] demonstrated that, as in malaria, the corrected NO concentration (corrected for renal impairment) in patients with severe leptospirosis is actually lower than in non-leptospirosis fever patients and patients with mild leptospirosis. The pathophysiological basis for this phenomenon remains elusive. Whether low NO levels contribute to pathogenesis of severe disease or whether it is a result of severe disease and acute kidney injury is unclear. Interestingly, there is an animal study by Pretre et al. [41] where infected mice and hamsters showed increased iNOS mRNA and protein in kidneys compared to controls. Giving the animals 4-aminopyridine, which is a iNOS inhibitor, caused faster deterioration. NO is one of the mediators which drives oxidative stress, and, like in many other diseases, oxidative stress is likely to play a role in tissue and organ

damage in leptospirosis, although currently evidence on this is limited.

Studies of immunochemical markers have shown that both cell mediated as well as humoral immunity are activated in severe leptospirosis. De Fost et al. [42], in an analysis of 44 Thai patients with definite or suspected leptospirosis, showed that markers of cell mediated immune activity was raised compared to healthy controls (Interferon [IFN]-gamma-inducible protein-10, granzyme B, monokine induced by IFN-gamma).

Proteomics seem to be a promising tool to study the inflammatory response in acute leptospirosis. The genomic sequences, despite being highly conserved among members of a species, do not show the functional status of a living cell (as genes are selectively turned on and off). Study of mRNA, though theoretically better to analyze gene expression, has many technical difficulties in practice (they are rapidly degraded, and not all mRNA are translated to proteins). In the light of these findings, the best way to assess the functional status of a cell is to assess its protein profile. However assessment of such profiles is complex, as these profiles change with time and from cell to cell depending on gene activation. Mass scale analysis of proteins in leptospirosis patients with severe disease has enabled identification of proteins that are differentially expressed in severe disease. Such identified proteins can be targets for further studies on pathogenesis and vaccine development [43,44]. In the most recently published study on proteomic analysis of serum of leptospirosis patients (compared to controls with malaria and healthy volunteers) Srivastava and colleagues [45] demonstrated several differentially expressed proteins in leptospirosis patients that were not previously associated with the disease pathogenesis. Therefore this is a rapidly evolving field.

Whether certain hosts of a particular genetic makeup have increased vulnerability to leptospirosis is of interest. A study by Fialho et al. [46] compared victims of leptospirosis with healthy controls for HLA alleles and genetic polymorphisms in the cytokine genes. Significant associations were found for certain alleles of HLA-A,B loci plus several HLA haplotypes. Polymorphisms in IL-4 and IL-4Rα genes were also significantly associated with a past history of leptospirosis. However, these findings have not been confirmed in larger population samples. Other studies have assessed different mediators of sepsis and cytokines in relation to severe leptospirosis. These mediators include human serum mannose binding lectin (which identifies pathogens activating the immune system) [47], soluble ST2 receptors, long pentraxin PTX3, copeptin and platelet activating factor acetylhydrolase (limited studies in animal models).

Membrane bound ST2 (mST2) is a negative regulator of toll like receptors (which is an important component of innate immunity). sST2 (soluble ST2) inhibits signaling via mST2. In an observational study in 68 severe leptospirosis patients, Wagenaar et al. [48] demonstrated that sST2 levels, cytokines IL-6, IL-8, and IL-10 were elevated in all patients. However sST2 levels had a significant association with any bleeding manifestation and severe bleeding. It also had a significant association with mortality (OR 2.4; 95% CI: 1.0-5.8). Interleukins 6 and 8 also showed a significant association with mortality but not with bleeding. In another study of assessing biomarkers of clinically severe leptospirosis, Wagenaar et al. [49] have shown that PTX3, a long pentraxin (pentraxins are a super family of large multimeric proteins that are thought play an important role in innate immunity and adjusting immune response) was elevated in leptospirosis and showed a significant association with mortality and disease severity. C-reactive protein is a structurally related protein (short pentraxin) but it did not show such a correlation with disease severity or death. In this study, both IL-6 and 8 were also shown to have a significant association with mortality. On the same cohort of patients, authors have also shown that copeptin (a stable peptide of arginine vasopressin precursor that is released in increased amounts in sepsis) levels were elevated in patients with severe leptospirosis and elevated levels were significantly associated with high mortality [50].

The study of biomarkers for severe disease has become more complex with recent genome wide studies in leptospira genome. Comparative analysis of saprophytic and pathogenic leptospira has shown that nearly 900 genes in pathogenic strains may be contributing to the pathogenicity of disease [51]. The functions of most of these genes are unknown and the known proteins which are thought to be of functional significance cannot explain all the virulence mechanisms of the organism. To make matters more complicated, it has been demonstrated that some of these genes are differentially regulated depending on the ambient conditions (temperature, osmolarity and iron levels). Mutation analysis systems have shown that some genes have definite roles in pathogenesis (as mutations in these genes attenuate virulence) and these include OmpA-family protein, Loa 22 and several other proteins [51].

Identified areas for further research in this fast developing field are; a) serial measurement of NOx levels in patients with leptospirosis to identify its use as a predictor of severity, b) further analysis of NOx with correction for creatinine released from muscle, c) further exploration of the role of oxidative stress in tissue and organ damage, d) use of cytokines as predictors of disease severity and e) proteomic analysis of sera of severe leptospirosis, mild leptospirosis, non leptospirosis fever patients (and healthy controls) on admission and serially to identify differentially expressed proteins that can be potential severity markers.

Conclusions

The ideal diagnostic test for leptospirosis should give a positive result as early as possible, should have good sensitivity and specificity plus be cost effective. MAT which is the presumed "gold standard" for leptospirosis is probably unsuitable for routine diagnosis due to its high false negative readings in early disease, lack of specificity for acute infection, and the cumbersomeness of the process. Other immunological methods such as immunochromatography and IgM ELISA have shown promise with early diagnosis and good sensitivities and specificities compared to MAT. Given the fact that MAT is may not be the ideal gold standard, Bayesian latent class models have shown that the sensitivities and specificities of these other tests may be higher than expected. Genomic diagnostics offer another exciting diagnostic possibility in early disease. However, the yields of these tests are low and they also need expensive equipment that is not freely available. Their use is currently limited to research and genotypic analysis.

The quest for a successful vaccine continues. The most efficient vaccines to-date are the whole cell killed vaccines which were also the earliest vaccines developed against leptospirosis. The disadvantage of these are that they are either monovalent or offers protection to a few locally circulating serovars. Research on subunit vaccines which offers universal protection against all pathogenic leptospira have not shown promising results despite having identified several proteins that are conserved among all pathogenic leptospira identified to-date.

Clinical features are not very good predictors of potential disease severity and therefore much of the recent focus in leptospirosis research is on identification of biomarkers that will predict severe disease in patients. Immunological studies have evaluated the role of cytokines such as IFN-γ, IL-6 and IL-8 in leptospirosis. Non specific activation of other cytokines such as TNF-α and IL-1 can increase the oxidative stress and free radicals. These may induce nitric oxide synthase activity resulting in higher total nitrite levels and overall reduced antioxidant capacity. They may have value as severity predictors. Genetic heterogeneity of HLA alleles, cytokine genes and proteomics of host and genomics of the pathogen are new ongoing avenues in research that might shed light in to having robust predictors for severe disease in future.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The review was conceptualized by CR who wrote the initial draft. SR supervised the project. All authors contributed to article search and information coding. All authors have read and approved the final manuscript.

Authors' information

SR (MD, FRCP, FRCPE, FACP, FCCP) is Professor in Medicine, and CR (MBBS, MD) is Lecturer in Medicine, Department of Clinical Medicine, Faculty of Medicine, University of Colombo, Sri Lanka. SH (MBBS, PhD) is Senior Lecturer at the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka. DF (MBBS, MD, PhD) is Professor in Parasitology, Faculty of Medicine, University of Colombo, Sri Lanka.

Author details

[1]Department of Clinical Medicine, Faculty of Medicine, University of Colombo, 25, Kynsey Road, Colombo 08, Sri Lanka. [2]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo, Sri Lanka. [3]Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka.

Received: 16 October 2014 Accepted: 22 December 2014
Published online: 16 January 2015

References

1. Pappas G, Papadimitriou P, Siozopoulou V, Christou L, Akritidis N. The globalization of leptospirosis: worldwide incidence trends. Int J Infect Dis. 2008;12(4):351–7.
2. Vijayachari P, Sugunan AP, Shriram AN. Leptospirosis: an emerging global public health problem. J Biosci. 2008;33(4):557–69.
3. Global burden of leptospirosis. Leptospirosis Burden Epidemiology Reference Group. [http://www.who.int/zoonoses/diseases/lerg/en/index2.html]. Accessed 15th January 2015.
4. Spichler AS, Vilaca PJ, Athanazio DA, Albuquerque JO, Buzzar M, Castro B, et al. Predictors of lethality in severe leptospirosis in urban Brazil. Am J Trop Med Hyg. 2008;79(6):911–4.
5. Chandrasekaran S, Gomathi S. A standard screening test for the early and rapid diagnosis of leptospirosis. Indian J Med Microbiol. 2004;22(1):23–7.
6. Sharma KK, Kalawat U. Early diagnosis of leptospirosis by conventional methods: one-year prospective study. Indian J Pathol Microbiol. 2008;51(2):209–11.
7. Boonyod D, Poovorawan Y, Bhattarakosol P, Chirathaworn C. LipL32, an outer membrane protein of Leptospira, as an antigen in a dipstick assay for diagnosis of leptospirosis. Asian Pac J Allergy Immunol. 2005;23(2–3):133–41.
8. Tahiliani P, Kumar MM, Chandu D, Kumar A, Nagaraj C, Nandi D. Gel purified LipL32: a prospective antigen for detection of leptospirosis. J Postgrad Med. 2005;51(3):164–8.
9. Coutinho ML, Vasconcellos FA, Fernandes CP, Seyffert N, Seixas FK, Ko AI, et al. Evaluation of the anti-LipL32 monoclonal antibodies potential for use in leptospirosis immunodiagnostic tests. J Immunoassay Immunochem. 2007;28(3):279–88.
10. 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(2):172–7.
11. Neves FO, Abreu PA, Vasconcellos SA, de Morais ZM, Romero EC, Nascimento AL. Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis. Arch Microbiol. 2007;188(5):523–32.
12. Natarajaseenivasan K, Vijayachari P, Sharma S, Sugunan AP, Selvin J, Sehgal SC. Serodiagnosis of severe leptospirosis: evaluation of ELISA based on the recombinant OmpL1 or LipL41 antigens of Leptospira interrogans serovar autumnalis. Ann Trop Med Parasitol. 2008;102(8):699–708.
13. Senthilkumar T, Subathra M, Phil M, Ramadass P, Ramaswamy V. Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay. Indian J Med Microbiol. 2008;26(1):45–9.
14. 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(1):73–81.
15. Aviat F, Rochereau-Roulet S, Branger C, Estavoyer JM, Chatrenet B, Orsonneau JL, et al. Synthetic peptide issued from Hap1/LipL32 for new early serodiagnosis of human leptospirosis. Comp Immunol Microbiol Infect Dis. 2010;33(5):375–87.
16. 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(5):289–97.
17. Lin X, Chen Y, Yan J. Recombinant multiepitope protein for diagnosis of leptospirosis. Clin Vaccine Immunol. 2008;15(11):1711–4.
18. 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 Sci. 2011;24(3):291–9.
19. Signorini ML, Lottersberger J, Tarabla HD, Vanasco NB. Enzyme-linked immunosorbent assay to diagnose human leptospirosis: a meta-analysis of the published literature. Epidemiol Infect. 2013;141(1):22–32.

20. Goris MG, Leeflang MM, Loden M, Wagenaar JF, Klatser PR, Hartskeerl RA, et al. Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. PLoS Negl Trop Dis. 2013;7(7):e2290.
21. 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(3):322–31.
22. Gravekamp C, Van de Kemp H, Franzen M, Carrington D, Schoone GJ, Van Eys GJ, et al. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiol. 1993;139(8):1691–700.
23. de Abreu FC, de Freitas VLT, Calo Romero E, Spinosa C, Arroyo Sanches MC, da Silva MV, et al. Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis. Trop Med Int Health. 2006;11(11):1699–707.
24. 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(2):247–57.
25. 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(1):e16236.
26. Agampodi SB, Matthias MA, Moreno AC, Vinetz JM. Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. Clin Infect Dis. 2012;54(9):1249–55.
27. Koizumi N, Watanabe H. Leptospirosis vaccines: past, present, and future. J Postgrad Med. 2005;51(3):210–4.
28. Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, et al. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine. 2007;25(33):6277–86.
29. Hartwig DD, Forster KM, Oliveira TL, Amaral M, McBride AJ, Dellagostin OA. A prime-boost strategy using the novel vaccine candidate, LemA, protected hamsters against leptospirosis. Clin Vaccine Immunol. 2013;20(5):747–52.
30. Seixas FK, Fernandes CH, Hartwig DD, Conceicao FR, Aleixo JA, Dellagostin OA. Evaluation of different ways of presenting LipL32 to the immune system with the aim of developing a recombinant vaccine against leptospirosis. Can J Microbiol. 2007;53(4):472–9.
31. Forster KM, Hartwig DD, Seixas FK, Bacelo KL, Amaral M, Hartleben CP, et al. A conserved region of leptospiral immunoglobulin-like (Lig) A and B proteins as a DNA vaccine elicits a prophylactic immune response against leptospirosis. Clin Vaccine Immunol. 2013;20(5):725–31.
32. Dellagostin OA, Grassmann AA, Hartwig DD, Felix SR, da Silva EF, McBride AJ. Recombinant vaccines against leptospirosis. Hum Vaccin. 2011;7(11):1215–24.
33. Cao Y, Faisal SM, Yan W, Chang YC, McDonough SP, Zhang N, et al. Evaluation of novel fusion proteins derived from extracellular matrix binding domains of LigB as vaccine candidates against leptospirosis in a hamster model. Vaccine. 2011;29(43):7379–86.
34. Grassmann AA, Felix SR, dos Santos CX, Amaral MG, Seixas Neto AC, Fagundes MQ, et al. Protection against lethal leptospirosis after vaccination with LipL32 coupled or coadministered with the B subunit of Escherichia coli heat-labile enterotoxin. Clin Vaccine Immunol. 2012;19(5):740–5.
35. Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, Romero EC, et al. Whole-genome analysis of Leptospira interrogans to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett. 2005;244(2):305–13.
36. Yan W, Faisal SM, McDonough SP, Chang CF, Pan MJ, Akey B, et al. Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. Vaccine. 2010;28(11):2277–83.
37. Atzingen MV, Vieira ML, Oliveira R, Domingos RF, Mendes RS, Barros AT, et al. Evaluation of immunoprotective activity of six leptospiral proteins in the hamster model of leptospirosis. Open Microbiol J. 2012;6:79–87.
38. Maciel EA, Athanazio DA, Reis EA, Cunha FQ, Queiroz A, Almeida D, et al. High serum nitric oxide levels in patients with severe leptospirosis. Acta Trop. 2006;100(3):256–60.
39. Gunaratna RI, Handunnetti SM, Bulathsinghalage MR, Somaratne P, Jayanaga A, de Silva HJ, et al. Serum nitrite levels in Sri Lankan patients with leptospirosis. Asian Pac J Trop Med. 2012;5(1):75–8.
40. Kalugalage T, Rodrigo C, Vithanage T, Somaratne P, De Silva HJ, Handunnetti S, et al. Low serum total nitrite and nitrate levels in severe leptospirosis. BMC Infect Dis. 2013;13(1):206.
41. Pretre G, Olivera N, Cedola M, Haase S, Alberdi L, Brihuega B, et al. Role of inducible nitric oxide synthase in the pathogenesis of experimental leptospirosis. Microb Pathog. 2011;51(3):203–8.
42. De Fost M, Chierakul W, Limpaiboon R, Dondorp A, White NJ, van Der Poll T. Release of granzymes and chemokines in Thai patients with leptospirosis. Clin Microbiol Infect. 2007;13(4):433–6.
43. Kositanont U, Saetun P, Krittanai C, Doungchawee G, Tribuddharat C, Thongboonkerd V. Application of immunoproteomics to leptospirosis: towards clinical diagnostics and vaccine discovery. Proteomics Clin Appl. 2007;1(4):400–9.
44. Thongboonkerd V. Proteomics in leptospirosis research: towards molecular diagnostics and vaccine development. Expert Rev Mol Diagn. 2008;8(1):53–61.
45. Srivastava R, Ray S, Vaibhav V, Gollapalli K, Jhaveri T, Taur S, et al. Serum profiling of leptospirosis patients to investigate proteomic alterations. J Proteomics. 2012;76 Spec No:56–68.
46. Fialho RN, Martins L, Pinheiro JP, Bettencourt BF, Couto AR, Santos MR, et al. Role of human leukocyte antigen, killer-cell immunoglobulin-like receptors, and cytokine gene polymorphisms in leptospirosis. Hum Immunol. 2009;70(11):915–20.
47. Miranda KA, Vasconcelos LR, Coelho LC, Lima Filho JL, Cavalcanti MS, Moura P. High levels of serum mannose-binding lectin are associated with the severity of clinical signs of leptospirosis. Braz J Med Biol Res. 2009;42(4):353–7.
48. Wagenaar JF, Gasem MH, Goris MG, Leeflang M, Hartskeerl RA, van der Poll T, et al. Soluble ST2 levels are associated with bleeding in patients with severe leptospirosis. PLoS Negl Trop Dis. 2009;3(6):e453.
49. Wagenaar JF, Goris MG, Gasem MH, Isbandrio B, Moalli F, Mantovani A, et al. Long pentraxin PTX3 is associated with mortality and disease severity in severe Leptospirosis. J Infect. 2009;58(6):425–32.
50. Limper M, Goeijenbier M, Wagenaar JF, Gasem MH, Isbandrio B, Kunde J, et al. Copeptin as a predictor of disease severity and survival in leptospirosis. J Infect. 2010;61(1):92–4.
51. Adler B, Lo M, Seemann T, Murray GL. Pathogenesis of leptospirosis: the influence of genomics. Vet Microbiol. 2011;153(1–2):73–81.

Performance Evaluations

CE

ISO 13485:2016

OTHER EVALUATIONS

LEPTOCHECK®-WB

Rapid test for the detection of IgM antibodies to Leptospirosis

KIT BIOMEDICAL RESEARCH

KONINKLIJK INSTITUUT VOOR DE TROPEN

ROYAL TROPICAL INSTITUTE

Zephyr Biomedicals
Dr. N. Sriram, Director
Plot Nos. M 46-47,
Phase III B, Verna Industrial Estate,
Verna, Goa - 403 722.
India

Date	*Extension*	*Enclosures*	*Your reference*
17 June 2003	+31 20 56 65438	1 results (1 page)	OB/PI/03-04/028
Subject			*Our reference*
evaluation rapid leptospirosis test			BR/ 162ims/03

Dear Dr. Sriram,

Please find enclosed our results from the evaluation of the Zephyr rapid test for leptospirosis. We used 3 tests to get familiar with the test. The evaluation is based on 47 tests.

We applied the test on sera from Dutch leptospirosis and non-leptospirosis cases. Results are compared with our MAT and IgM ELISA as well as with the rapid tests developed at this department.

Based on these results the Zephyr test performs well.

If you have any question, please do not hesitate to contact me.

Yours sincerely,

KIT Biomedical Research

Dr. Rudy A. Hartskeerl
Head Leptospirosis Reference Centre (WHO/FAO/OIE/RIVM)

cc. Dr. Avinash Tulaskar

Meibergdreef 39

1105 AZ AMSTERDAM
The Netherlands

Phone
+31 20 56 65441
Fax
+31 20 6971841

E-mail
R.Hartskeerl@kit.nl
Internet
www.kit.nl

Bank
ABN AMRO 40 50 05 970
ABN AMRO USD 62 62 48 183
Postbank 24548

Overwiew comparison Rapid test for Leptospirosis, Zephyr Biomedicals (India) with diagnostic tests performed at Royal Tropical Institute (KIT), Amsterdam, June 2003

labnr.	Initial	days of illness	ELISA (reciproque titers)	highest MAT titer (reciproque titers)	Leptotek Lateral Flow	DriDot test (sec.)	Grippo. ELISA (reciproque titers)	infecting serogroup	Rapid Test of Zephyr Biomedicals Read by 4 people: H.Sm.	M.E.	M.G	R.H
5481	A	-	<10	<20	neg	neg			neg	neg	neg	neg
5469	Ba	31	<10	20	neg	neg			neg	neg	neg	neg
5236	Bl	27	40	80	2+	1	160	Grippotyphosa	neg	df	neg	neg
5260	Bl	34	<10	320	2+	3	640	Grippotyphosa	2+	2+	2+	1+
5435	Bk	-	<10	<20	neg	neg			neg	neg	neg	neg
5280	Boe	7	160	320	2+	4		Icterohaemorrhagiae	1+	df	df	df
5436	Bou	16	40	20	neg	8			neg	neg	neg	neg
5242	D	11	160	320	1+	3	640	Grippotyphosa	df	df	df	neg
5349	D	56	40	40	2+	8	160	Grippotyphosa	df	df	1+	df
5438	F	-	<10	20	neg	neg			neg	neg	neg	neg
5468	G	137	<10	<20	neg	neg			neg	neg	neg	neg
5172	Ha	4	<10	<20	neg	0		Icterohaemorrhagiae	neg	neg	df	neg
5188	Ha	7	<10	40	2+	30		Icterohaemorrhagiae	2+	1+	1+	1+
5241	Ha	27	40	1280	1+	4		Icterohaemorrhagiae	df	df	df	df
5471	He	19	<10	20	df	neg			neg	neg	neg	neg
5327	Ho	13	<10	40	1+	10		Pomona	1+	df	1+	1+
5335	Ho	24	<10	80	2+	8		Pomona	df	df	df	df
5470	Ke	6	<10	<20	neg	neg			neg	neg	neg	neg
5136	Ko	10	<10	40	2+	15	40	Grippotyphosa	df	df	df	neg
5161	Ko	23	40	40	1+	3	160	Grippotyphosa	1+	1+	1+	1+
5268	Ko	54	80	20	2+	10	80	Grippotyphosa	1+	1+	1+	df
5463	Ks	-	<10	<20	neg	neg			neg	neg	neg	neg
5448	Kr	29	40	<20	neg	20			neg	neg	neg	neg
5273	La	2	<10	<20	df	neg		Icterohaemorrhagiae	neg	neg	neg	neg
5355	La	39	40	320	2+	4		Icterohaemorrhagiae	1+	1+	1+	1+
6338	La	27	80	80	2+	5		Icterohaemorrhagiae	1+	1+	1+	1+
5231	Ln	9	320	640	2+	2		Icterohaemorrhagiae	2+	2+	2+	2+
5285	Ln	24	160	5120	2+	2		Icterohaemorrhagiae	2+	1+	1+	1+
5475	Lk	19	<10	20	neg	neg			neg	neg	neg	neg
5426	Le	-	<10	<20	neg	neg			neg	neg	neg	neg
5288	Li	4	1280	1280	2+	3		Icterohaemorrhagiae	2+	2+	1+	1+
5320	Li	19	1280	10240	1+	8		Icterohaemorrhagiae	1+	1+	1+	1+
5467	Me	-	<10	<20	neg	neg			neg	neg	neg	neg
5439	Mo	-	<10	20	neg	neg			neg	neg	neg	neg
5430	N	10	160	80	2+	10		Pomona	2+	2+	2+	1+
5445	N	18	160	2560	2+	4		Pomona	1+	df	1+	1+
5472	N	41	80	1280	2+	26		Pomona	1+	df	df	df
5182	O	1	40	80	2+	df		Icterohaemorrhagiae	2+	1+	1+	1+
5227	O	14	320	2560	2+	2		Icterohaemorrhagiae	df	df	df	1+
5432	P	2	<10	<20	df	neg			neg	neg	neg	neg
5441	S	5	<10	<20	df	neg			neg	neg	neg	df
5459	Ti	24	<10	20	neg	neg			neg	neg	neg	df
5314	Tw	15	160	160	2+	14		Icterohaemorrhagiae	2+	2+	1+	1+
5440	V	15	<10	20	neg	neg			neg	neg	neg	neg
5208	W	5	<10	<20	neg	neg		Icterohaemorrhagiae	neg	neg	neg	neg
5244	W	13	80	640	2+	3		Icterohaemorrhagiae	2+	2+	2+	1+
5325	W	41	40	320	2+	16		Icterohaemorrhagiae	2+	2+	2+	2+

Sera with the same initials are convalescent sera
We differentiated the results in: Negative: no band, doubtful: very weak band, 1+: visible band, 2+: strong band
df means doubtful

OPERATION LEPTOSPIRA

UMC UTRECHT

GENERAL INFORMATION

Leptospirosis (Weil's disease, Mud Fever, Melkers Fever) is a global common bacterial zoonosis.There are various pathogenic serovars with different specific hosts. Transmission takes place via the urine of the host, usually mice, rats and bovines. In humans, the disease is characterized by fever, malaise, muscle and joint pain, photophobia. Also jaundice, hepatic and renal impairment and meningitis occur. An important part of the infections in the Netherlands has risen abroad and heavily identified with contact with open water. The diagnosis is made by demonstrating the presence of Leptospira spp. itself or antigens thereof, or specific antibodies against Leptospira spp.The choice of the diagnostic test is also dependent on the duration of illness. At a duration of disease of 10 days or less has a PCR on EDTA blood, and / or urine are preferred. Optionally, a culture can be used on these materials. To apply for a culture or PCR must first be discussed with the officiating virologist associated with specific purchase and transport conditions.

In addition, for the presence of IgM antibodies against Leptospira spp. be tested. This happens in the virology laboratory through the quick test. A positive rapid test is standard for confirmation using an ELISA and / or the microscopic agglutination test (MAT) sent to the Leptospirosis Reference Laboratory in Amsterdam. Negative results and a lasting suspicion of a recent history of infection should be considered to repeat the serology after 2 weeks.

With a disease duration of 11 days or longer must be requested only serology. For this, the rapid test that is carried out in the virology laboratory suitable. The sensitivity of serology in serum decreased from 11 days after the onset of the symptoms is high as well as the negative predictive value. The rapid test can be performed if necessary in consultation with the attending virologist CITO.

DELIVERY ADDRESS MATERIALS

UMC Utrecht, Medical Microbiology
Counter 22 House Postnummer G.04.427
Heidelberglaan 100
3584 CX Utrecht

Determination	**Grow on leptospira**
Technique	Grow
Indication	Detection of an infection with Leptospira spp. in a disease duration of 10 days or less
Material	Heparin blood / urine / CSF
Required volume	3-10 ml
Result	Negative / Positive
Comments	This is a provision to send the Leptospirosis Reference Laboratory in Amsterdam. In general, a PCR assay for the detection of Leptospira in preference to the culture. Submission materials only after consultation with the dd. virologist associated with specific shipping instructions.
Information / contact	Administration 088 75 588 29 or dd clinical microbiologist virology 71762
Determination	**Leptospira DNA**

Technique	PCR
Indication	Detection of an infection with Leptospira spp. in a disease duration of 10 days or less
Material	EDTA whole blood (not plasma) / serum / CSF / urine
Required volume	5 ml
Result	Negative / Suspect / Positive
Comments	This is a provision to send the Leptospirosis Reference Laboratory in Amsterdam. Submit material after consultation with the dd. Virologist
Information / contact	Administration 088 75 588 29 or dd clinical microbiologist virology 71762
Determination	**Leptospirosis rapid test**
Technique	immuunchromatografische test Leptocheck-WB Zephyr Biomedicals
Indication	detect specific antibodies against leptospira
Material	serum
Required volume	5 ml
Inzetdag	every day
Rash known	within 1 day
Result	positive, negative
Comments	If material is material is sent for confirmation to the rapid test positive for the Leptospirosis Reference Laboratory in Amsterdam
Information / contact	Administration 088 75 588 29 or dd clinical microbiologist virology 71762
Determination	**Microscopic Agglutination Test (MAT)**
Technique	agglutination
Indication	confirmation determination of a positive rapid test
Material	serum, plasma (EDTA)
Required volume	5 ml

Inzetdag	Cito after consultation with dd virologist
Result	positive, negative, specifically serovar
Comments	This is a provision to send the Leptospirosis Reference Laboratory in Amsterdam
Information / contact	Administration 088 75 588 29 or dd clinical microbiologist virology 71762

For further information contact :

TULIP DIAGNOSTICS (P) LTD

Gitanjali, Tulip Block, Dr. Antonio Do Rego Bagh, Alto Santacruz, Bambolim Complex Post Office, Goa - 403202, INDIA.

Tel.: +91 832 2458546-50 **Fax :** +91 832 2458544 **E-mail :** sales@tulipgroup.com **Website :** www.tulipgroup.com