Performance Evaluations

LEPTOCHECK®-WB
Rapid test for the detection of IgM antibodies to leptospirosis
<table>
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<tr>
<th>S. No.</th>
<th>Name of the Publication</th>
<th>Pg Nos</th>
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<tr>
<td>1.</td>
<td>International Journal of Research in Pharmaceutical and Biomedical Sciences,</td>
<td>412-416</td>
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<td>Vol. 4 (2) Apr– Jun 2013</td>
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<td>3.</td>
<td>National Journal Of Community Medicine 2011 Volume 2 Issue 1</td>
<td>64-70</td>
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<td><a href="http://www.aissl.org/ABSTRACTS-Basic%20Immunology.pdf">www.aissl.org/ABSTRACTS-Basic%20Immunology.pdf</a></td>
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### OTHER EVALUATIONS

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<tr>
<td>12.</td>
<td>Royal Tropical Institute (KIT) Amsterdam, June 2003</td>
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<td>13.</td>
<td>Leptospirosis Reference Laboratory, Amsterdam</td>
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Reference Articles on Google Books

Issues in Medical Microbiology, Mycology, Virology, and Molecular Medicine, Pg 26.
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 *Leptospirotsacterohemorrhagiae*. 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 IgM antibodies 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 IgM ELISA, 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, Leptospirotsacterohemorrhagiae, Fletcher’s medium.

INTRODUCTION

Leptospirosis is an endemic and epidemic febrile disease. It has 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. 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. 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. 

Fever was the most common symptoms seen in 100% patients. This is consistent with the study conducted by Deyet al. Data published worldwide
shows the incidence of icteric manifestations ranging from 16% to 100%. In our study, it was 66% and similar to the other study conducted in Mumbai by Dey et al.

Following significant laboratory findings were observed in leptochek 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 were detected 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%.

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 *Leptospira* 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 panbiolgM 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 distribution of Leptospirosis (n=50)**

<table>
<thead>
<tr>
<th></th>
<th>PAEDIATRICS No. (%)</th>
<th>ADULT No. (%)</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically suspected patients</td>
<td>03 (6%)</td>
<td>47 (94%)</td>
<td>50</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>MALES No. (%)</th>
<th>FEMALES No. (%)</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically suspected patients</td>
<td>43 (86%)</td>
<td>7 (14%)</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 3: Clinical manifestations and correlation with Leptocheck test. (n=50)

<table>
<thead>
<tr>
<th>SYMPTOMS AND SIGNS</th>
<th>No. (%)</th>
<th>Leptospirosis Sero +</th>
<th>Leptocheck Sero + (n=15)</th>
<th>Leptocheck Negative (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYMPTOMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever with chills</td>
<td>50 (100%)</td>
<td>15 (100%)</td>
<td>35 (100%)</td>
<td></td>
</tr>
<tr>
<td>Head ache</td>
<td>15 (30%)</td>
<td>7 (46.7%)</td>
<td>8 (22.9%)</td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td>34 (68%)</td>
<td>12 (80%)</td>
<td>22 (62.9%)</td>
<td></td>
</tr>
<tr>
<td>Myalgia</td>
<td>25 (50%)</td>
<td>7 (46.7%)</td>
<td>18 (51.4%)</td>
<td></td>
</tr>
<tr>
<td>Haematemesis</td>
<td>1 (2%)</td>
<td>1 (6.7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Anuria</td>
<td>3 (6%)</td>
<td>3 (20.0%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SIGNS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp. &gt;= 100°C</td>
<td>32 (64%)</td>
<td>15 (100%)</td>
<td>17 (48.6%)</td>
<td></td>
</tr>
<tr>
<td>Subconjunctival Suffusion</td>
<td>3 (6%)</td>
<td>3 (20.0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Jaundice</td>
<td>15 (30%)</td>
<td>10 (66.7%)</td>
<td>5 (14.3%)</td>
<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>12 (24%)</td>
<td>7 (46.7%)</td>
<td>5 (14.3%)</td>
<td></td>
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<tr>
<td>Petechial Haemorrhage</td>
<td>1 (2%)</td>
<td>1 (6.7%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Hemoptysis (ARDS)</td>
<td>1 (2%)</td>
<td>1 (6.7%)</td>
<td>0</td>
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</table>

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

<table>
<thead>
<tr>
<th>LABORATORY PARAMETERS</th>
<th>NORMS</th>
<th>Leptocheck Positive (n=15)</th>
<th>Leptocheck Negative (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Hb % (&lt; 11 gm%)</td>
<td>M 14 – 18 gm F 12 – 16 gm</td>
<td>1(6.7%)</td>
<td>1(2.9%)</td>
</tr>
<tr>
<td>High TLC (Leukocytosis) &gt; 11000 (mcu mm³)</td>
<td>5000 – 11000 (mcu mm³)</td>
<td>9(60.0%)</td>
<td>26(74.3%)</td>
</tr>
<tr>
<td>Low Platelet count &lt; 1.5 lac /cu mm</td>
<td>1.5 – 4.0 lac /cu mm</td>
<td>11(73.3%)</td>
<td>1(2.9%)</td>
</tr>
<tr>
<td>High bilirubin &gt; 1 mg/dl</td>
<td>0.3 – 1.0 mg/dl</td>
<td>12(80.0%)</td>
<td>13(37.1%)</td>
</tr>
<tr>
<td>High SGPT 0 – 35 IU/L</td>
<td>0 – 35 IU/L</td>
<td>12(80.0%)</td>
<td>19(54.3%)</td>
</tr>
<tr>
<td>High SGOT 0 – 35 IU/L</td>
<td>0 – 35 IU/L</td>
<td>12(80.0%)</td>
<td>21(63.0%)</td>
</tr>
<tr>
<td>High Alkaline Phosphatase 36 – 141 IU/L</td>
<td>21(13.3%)</td>
<td>2(6.0%)</td>
<td></td>
</tr>
<tr>
<td>High BUN 10 – 20 mg/dl</td>
<td>10 – 20 mg/dl</td>
<td>12(80.0%)</td>
<td>17(48.6%)</td>
</tr>
<tr>
<td>High Serum creatinine 0.5 – 1.5 mg/dl</td>
<td>0.5 – 1.5 mg/dl</td>
<td>6(40.0%)</td>
<td>8(22.9%)</td>
</tr>
<tr>
<td>Urine R/M Alb/Hematuria/Pyuria</td>
<td>10(66.7%)</td>
<td>1(2.9%)</td>
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</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Blood DGM</th>
<th>Urine DGM(Suspected)</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>5(10%)</td>
<td>3(6%)</td>
<td>3(6%)</td>
</tr>
</tbody>
</table>

Table 6: Positive serological results in Leptospira suspected patients (n=50)

<table>
<thead>
<tr>
<th>IgM (Lepto Check)</th>
<th>ELISA</th>
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<tbody>
<tr>
<td>15(30%)</td>
<td>13 (26%)</td>
</tr>
</tbody>
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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
Comparision between Leptocheck IgM & ELISA

Fig. 5: Showing comparison between Leptocheck and IgM ELISA

REFERENCES
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%), icterohaemorrhagiae (8%) and grippotyphosa (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 transmitted to humans through the environment or direct contact with urine from infected animals. Infections with pathogenic Leptospira are increasingly recognized as a common cause of acute febrile illness in tropical environments. 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. It is most common in tropical countries like Nicaragua, India and Thailand. Pulmonary involvement in leptospirosis was first observed in India during outbreaks in Andaman Islands. In Australia also pulmonary hemorrhage has been reported in patients with leptospirosis. 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. 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.

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, conjunctival 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 44.4°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 drinking water for four days and four nights. The people were trapped in their houses without food and water. 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 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 conjunctival 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), agryphosa (MoskvaV), canicola (HondUtrechIV), hebdomadis (Hebdomadis), pomona (Pomona), patoc (PatocI), pyrogenes (Perepelcian), 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 Microbiology, 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 ELSA (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 Leptochek (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

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

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

<table>
<thead>
<tr>
<th>Age</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-9</td>
<td>17</td>
<td>11</td>
<td>28</td>
<td>2.22</td>
</tr>
<tr>
<td>10-19</td>
<td>164</td>
<td>74</td>
<td>238</td>
<td>18.91</td>
</tr>
<tr>
<td>20-29</td>
<td>214</td>
<td>144</td>
<td>358</td>
<td>28.45</td>
</tr>
<tr>
<td>30-39</td>
<td>189</td>
<td>192</td>
<td>382</td>
<td>30.28</td>
</tr>
<tr>
<td>40-49</td>
<td>64</td>
<td>30</td>
<td>94</td>
<td>7.47</td>
</tr>
<tr>
<td>50-59</td>
<td>51</td>
<td>41</td>
<td>92</td>
<td>7.31</td>
</tr>
<tr>
<td>60-69</td>
<td>44</td>
<td>12</td>
<td>56</td>
<td>4.45</td>
</tr>
<tr>
<td>70-79</td>
<td>9</td>
<td>7</td>
<td>16</td>
<td>1.27</td>
</tr>
<tr>
<td>80-89</td>
<td>11</td>
<td>3</td>
<td>14</td>
<td>1.11</td>
</tr>
</tbody>
</table>

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 (30) 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

<table>
<thead>
<tr>
<th>Area</th>
<th>No. of cases (%)</th>
<th>Relative risk</th>
<th>Death reported</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surat</td>
<td>1103 (87.6)</td>
<td>33.50</td>
<td>85</td>
<td>6.75</td>
</tr>
<tr>
<td>Navsari</td>
<td>110 (8.74)</td>
<td>19.30</td>
<td>29</td>
<td>2.30</td>
</tr>
<tr>
<td>Valsad</td>
<td>45 (3.57)</td>
<td>1.0</td>
<td>7</td>
<td>0.55</td>
</tr>
<tr>
<td>Total</td>
<td>1258 (100)</td>
<td></td>
<td>121</td>
<td>9.61</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Months</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>October</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surat</td>
<td>22</td>
<td>453</td>
<td>614</td>
<td>14</td>
</tr>
<tr>
<td>Navsari</td>
<td>21</td>
<td>54</td>
<td>31</td>
<td>04</td>
</tr>
<tr>
<td>Valsad</td>
<td>07</td>
<td>14</td>
<td>21</td>
<td>03</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>521</td>
<td>666</td>
<td>21</td>
</tr>
</tbody>
</table>
The predominant serovars encountered for the outbreak was determined by MAT. Serovars like *autumnalis* (40%), *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 evaluated 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

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumnalis</td>
<td>357</td>
<td>46</td>
</tr>
<tr>
<td>Australis</td>
<td>298</td>
<td>38</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>238</td>
<td>30</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>66</td>
<td>8</td>
</tr>
<tr>
<td>Cynopteri</td>
<td>158</td>
<td>20</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>13</td>
<td>1.6</td>
</tr>
<tr>
<td>Patoc</td>
<td>13</td>
<td>1.6</td>
</tr>
</tbody>
</table>

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

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

### 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, when thousands of patients developed acute undifferentiated febrile illness and several dozen died of severe pulmonary hemorrhages as the predominant signs and symptoms. 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. 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 conjunctival 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 exhibit from previous reports as Australis and Autumnalis usually accompanied by the symptoms like fever, myalgia, and nausea and vomiting, jaundice like signs, conjunctival suffusion and haemorrhagic 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 subsist from previous reports as Australis and Autumnalis. The serovars of Leptospira interrogans isolated from cases of 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 in 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 epidemiologic 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

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

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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%, 98.36%, 92.37% and 0.81 in acute phase of disease. Corresponding values for IgM ELISA were 96.82%, 88.05%, 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%, 95.65%, 96.96%, 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 distribution1. Humans become infected through contact with contaminated animal urine, tissues, or water2 The clinical presentation is difficult to distinguish from dengue, malaria, influenza, and many other diseases characterized by fever, headache, and myalgia3. 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 surveillance4. 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. 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. 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), an IgM dipstick assay (LDS), an IgM dot-ELISA dipstick test (DST), and the indirect haemagglutination assay (IHA). Reported evaluations suggest that some of these assays are highly sensitive and specific, 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:

• Conjunctival 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. 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°C 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 >80 and positive at titre of >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 caliber 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 ≥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

<table>
<thead>
<tr>
<th>Tests</th>
<th>Phases</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptocheck</td>
<td>Acute Phase (&lt; 14 days)</td>
<td>98.36%</td>
<td>86.95%</td>
<td>86.95%</td>
<td>98.36%</td>
<td>92.37%</td>
</tr>
<tr>
<td>WB</td>
<td>Convalescent phase (14-28 days)</td>
<td>87.87%</td>
<td>88.00%</td>
<td>90.62%</td>
<td>84.61%</td>
<td>6.20%</td>
</tr>
<tr>
<td>IgM ELISA</td>
<td>Acute Phase (&lt; 14 days)</td>
<td>96.82%</td>
<td>88.05%</td>
<td>88.40%</td>
<td>96.72%</td>
<td>91.53%</td>
</tr>
<tr>
<td></td>
<td>Convalescent phase (14-28 days)</td>
<td>91.42%</td>
<td>95.62%</td>
<td>96.96%</td>
<td>88.00%</td>
<td>93.10%</td>
</tr>
</tbody>
</table>

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 exposure. 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

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
<th>WYsekhar EH Soo⁴,⁸</th>
<th>SC Sehgal, PV Vijaychari⁵,⁷</th>
<th>Present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid test</td>
<td>Sensitivity</td>
<td>83.3%</td>
<td>78.7%</td>
<td>93.81%</td>
</tr>
<tr>
<td>Leptocheck or Dipstick</td>
<td>Specificity</td>
<td>93.8%</td>
<td>88.3%</td>
<td>86.81%</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>95.29%</td>
<td>91.0%</td>
<td>88.34%</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>79%</td>
<td>73.4%</td>
<td>92.94%</td>
</tr>
<tr>
<td>IgM ELISA</td>
<td>Sensitivity</td>
<td>54.2%</td>
<td>78.5%</td>
<td>93.81%</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>96.9%</td>
<td>87.6%</td>
<td>90.10%</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>96.3%</td>
<td>90.5%</td>
<td>91.00%</td>
</tr>
<tr>
<td></td>
<td>NPV</td>
<td>58.5%</td>
<td>73%</td>
<td>93.81%</td>
</tr>
</tbody>
</table>

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, Soo²⁰, P. Vijayachari et al²¹). 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 al²¹ & W.Y. Sekhar EH Soo²⁰ 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


ABSTRACT

Introduction: Leptospirosis is an acute febrile disease, in tropical and sub-tropical regions of the 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.

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 (Bangkok1), L.Ballum (Ballum), L.Serovar (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, Port Blair. These serovars were maintained in semisolid 0.1% EMJH (Ellingshausen-McCullough-Johnson-Harris) agar by using Leptospira medium base supplemented with 10% enrichment (Difco,USA) at 28-30°C. 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 leptosporial 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 LeptospiralG 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 LeptospiralG/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 WB and Latex agglutination test showed sensitivities of 91.0%, 88.1% and 72.7% & specificities of 78.9%, 65.6% and 45.6% respectively as compared to MAT. 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].

DISCUSSION
As the disease Leptospirosis shows protean clinical manifestations, laboratory confirmation is a must. Isolation of leptospires 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
Leptospirosis IgM/IgG was closer to that of the MAT. Considering MAT as reference and compared with various rapid tests, SD leptospiro 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 leptospiro 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 leptospioral 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, sensitive and specific to 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

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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


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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 enzyme linked immunosorbent assay (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 Leptocheck-WB test (LCT) against the microscopic agglutination test (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 further investigated.

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

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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 IgM 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 km² 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, conjunctival 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 \( L.\, b.\, f.\, a.\, x.\, a.\) 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 \( L.\, b.\, f.\, a.\, x.\, a.\) 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 \( L.\, b.\, f.\, a.\, x.\, a.\) organisms, was considered as the reporting titer. Single acute MAT positivity was defined as a titer of ≥400. Final MAT positivity was defined as a titer of ≥400 in single sample, sero-conversion from negative to a titer ≥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 \( L.\, b.\, f.\, a.\, x.\, a.\) 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 ≥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\SerionGmbH, 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 (≥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 ≥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 ≥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.

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

doi:10.1371/journal.pone.0129236.t001
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.
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% (95.3%-99.2%), 84.3 (80.3%-87.7%) 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.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample size</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM-ELISA (Virion/Serion)</td>
<td>Panwala et al [13]</td>
<td>130</td>
<td>93.8</td>
</tr>
<tr>
<td></td>
<td>Kucerova et al [33]</td>
<td>45</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Effler et al [34]</td>
<td>344</td>
<td>48.0</td>
</tr>
<tr>
<td></td>
<td>Present study</td>
<td>888</td>
<td>86.9</td>
</tr>
<tr>
<td>Leptocheck WB test</td>
<td>T Panwala [13]</td>
<td>130</td>
<td>93.8</td>
</tr>
<tr>
<td></td>
<td>MG Goris [35]</td>
<td>197</td>
<td>78.0</td>
</tr>
<tr>
<td></td>
<td>Present study</td>
<td>888</td>
<td>86.1</td>
</tr>
</tbody>
</table>

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 (Institut Virion\SerionGmbH, 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


Performance Evaluations

AS A REFERENCE PRODUCT

LEPTOCHECK®-WB
Rapid test for the detection of IgM antibodies to leptospirosis
A study on few biochemical parameters of clinically suspected and laboratory confirmed Leptospirosis cases

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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 leptospirosis positive patient 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; Mamuthaetupathi 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 region ‘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
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 were 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.

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

<table>
<thead>
<tr>
<th>Function</th>
<th>Parameters</th>
<th>Elevated value in Leptospirosis positive patient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>SGOT (U/L)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SGPT (U/L)</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Bilirubin (mg/dL)</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Creatinine (mg/dL)</td>
<td>89</td>
</tr>
<tr>
<td>Kidney</td>
<td>Blood Urea (mg/dL)</td>
<td>49</td>
</tr>
</tbody>
</table>

**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**


Molecular characterisation and disease severity of leptospirosis in Sri Lanka

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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 4°C 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 (Leptochek 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-
Carried out using PCR primers: rrs-outer F (5′-CTCAACCTGTTCTCTAAAGTTCAAC, 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 94°C for 5 min followed by 45 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 10 min and a final elongation step at 72°C for 10 min with final hold at 4°C. The resulting amplicon was 793 bp and these were stored at 4°C 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 37°C 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, Interrogans serovar Pyrogenes. An undigested PCR product, where the reaction mixture was prepared without Hind III, Hae III 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′-CTCA-GAACCTGCTTGCGGC-3′), rrs-outer-R (5′-TCTCTACCTGTTTACCCCATC-3′), rrs-inner-F (5′-CTGGGCCTCTGCGG-3′), rrs-inner-R (5′-GGTTTACCTCCTGTCGA-3′) (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 MgCl₂ (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 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 67°C for 15 s, 72°C for 30 s, another 40 cycles of 95°C for 10 s, 55°C for 15 s, 72°C for 30 s and a final elongation step at 72°C 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 Leptospira Patoc 1 strain; 7-13: flaB PCR positive patient samples.

**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
**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.

<table>
<thead>
<tr>
<th>Category (Leptospira case definition)</th>
<th>Method</th>
<th>Result</th>
<th>Patients n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definitive cases</td>
<td>MAT</td>
<td>+</td>
<td>61 (36)</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>+</td>
<td>14 (8.3)</td>
</tr>
<tr>
<td></td>
<td>MAT and PCR</td>
<td>+</td>
<td>7 (4.2)</td>
</tr>
<tr>
<td></td>
<td>MAT or PCR</td>
<td>+</td>
<td>66 (39.2)</td>
</tr>
<tr>
<td></td>
<td>MAT, PCR and Leptocheck</td>
<td>+</td>
<td>6 (3.6)</td>
</tr>
<tr>
<td>Presumptive cases</td>
<td>Leptocheck</td>
<td>+</td>
<td>84 (50)</td>
</tr>
<tr>
<td>Unconfirmed cases</td>
<td>MAT, PCR and Leptocheck</td>
<td>-</td>
<td>73 (43.4)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>-</td>
<td>-</td>
<td><strong>168 (100)</strong></td>
</tr>
</tbody>
</table>

\(a\): single sample MAT \(\geq 1:400\); - negative; +: positive.

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

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 three 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 III digestion resulted in three DNA fragments 100 bp, 300 bp and 350 bp in all reference strains; *L. interrogans* serovar Canicola, Icterohaemorrhagiae 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...
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 \textit{L. interrogans} and 25% had \textit{L. borgpetersenii} infection. Renal failure was seen in 35.7% of the confirmed cases out of them, 80% were due to \textit{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,
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 II digestion was more discriminative than Hind III 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

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**TABLE II**

*Leptospira* sequence identity related to disease complications

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

---

**TABLE III**

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

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

a: Agampodi et al. (2011); b: Agampodi et al. (2014); c: current study; IQR: interquartile range.
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 brounii, Leptospira inadai, Leptospira licerasiae, Leptospira wolffi 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 socioeconomic 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.

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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 Harugoda, Faculty of Medicine, University of Kelaniya, for providing serological diagnostic kits.

REFERENCES


Leptospirosis- A Physician’s dilemma or diagnostic enigma?

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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 Microscopic 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.[³⁴] 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
Peripheral smear for Malaria parasite: Negative

Urine Routine and Microscopy: No abnormality detected

Blood culture: Sterile

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 leptospiro 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,
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.\cite{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).\cite{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.\cite{1,3,4}

However, both serum samples were negative by MAT which is considered as serological gold standard for the diagnosis of leptospirosis.\cite{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.\cite{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.\cite{11} In India, earlier sporadic human case reports with *Leptospira inadai* infection and circulation of *Leptospira inadai* in reservoir hosts have been reported.\cite{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)**
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References:
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Assessment of oxidative stress in severe leptospirosis patients

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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 (NFLF) controls (n=41) and a healthy control group (n=20) was also included. NO\textsuperscript• was measured by determining serum NO\textsubscript{2-} and NO\textsubscript{x} (NO\textsubscript{2-}+NO\textsubscript{x}) 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 NO\textsubscript{2-}levels compared to NFLF and healthy controls (1.8±0.11 μM, 1.2±0.08 μM and 1.1±0.08 μM respectively; P<0.001). Serum NO\textsubscript{x} levels (18.5±1.50 μM) of severe patients were significantly higher compared to leptospirosis patients, NFLF and healthy control groups (14.3±0.94 μM, 12.3±0.89 μM and 5.5±0.27 μM; P<0.05 respectively). Both severe and mild leptospirosis 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 NFLF and healthy control groups (0.97±0.04 and 0.92±0.03 μM/mg protein; P<0.05 respectively). Thus, serum NO\textsubscript{x} could be used as a prognostic indicator of severity of leptospirosis.

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

Senaka Rajapakse1*, Chaturaka Rodrigo1, Shiroma M Handunnetti2 and Sumadhya Deepika Fernando3

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
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 diagnostic testing, 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 leptospirosis 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 up to 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 leptosira. 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 leptospiiraemia 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 leptosira 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 leptospiira 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.

Leptospiiral 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 leptosira 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
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 creates a barrier in correlating clinical features with the infecting species.

Molecular markers of severe leptospirosis

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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-4Ra genes were also significantly associated with the disease pathogenesis. 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 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

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Author details

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References


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
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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

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<td>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.</td>
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<td>Comments</td>
<td>This is a provision to send the Leptospirosis Reference Laboratory in Amsterdam. Submit material after consultation with the dd. Virologist</td>
</tr>
</tbody>
</table>

| Information / contact | Administration 088 75 588 29 or dd clinical microbiologist virology 71762 |

<table>
<thead>
<tr>
<th>Determination</th>
<th>Leptospirosis rapid test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technique</td>
<td>immunochromatografische test Leptocheck-WB Zephyr Biomedicals</td>
</tr>
<tr>
<td>Indication</td>
<td>detect specific antibodies against <em>leptospira</em></td>
</tr>
<tr>
<td>Material</td>
<td>serum</td>
</tr>
<tr>
<td>Required volume</td>
<td>5 ml</td>
</tr>
<tr>
<td>Inzetdag</td>
<td>every day</td>
</tr>
<tr>
<td>Rash known</td>
<td>within 1 day</td>
</tr>
<tr>
<td>Result</td>
<td>positive, negative</td>
</tr>
<tr>
<td>Comments</td>
<td>If material is material is sent for confirmation to the rapid test positive for the Leptospirosis Reference Laboratory in Amsterdam</td>
</tr>
</tbody>
</table>

| Information / contact | Administration 088 75 588 29 or dd clinical microbiologist virology 71762 |

<table>
<thead>
<tr>
<th>Determination</th>
<th>Microscopic Agglutination Test (MAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technique</td>
<td>agglutination</td>
</tr>
<tr>
<td>Indication</td>
<td>confirmation determination of a positive rapid test</td>
</tr>
<tr>
<td>Material</td>
<td>serum, plasma (EDTA)</td>
</tr>
<tr>
<td>Required volume</td>
<td>5 ml</td>
</tr>
<tr>
<td><strong>Inzetdag</strong></td>
<td>Cito after consultation with dd virologist</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td><strong>Result</strong></td>
<td>positive, negative, specifically serovar</td>
</tr>
<tr>
<td><strong>Comments</strong></td>
<td>This is a provision to send the Leptospirosis Reference Laboratory in Amsterdam</td>
</tr>
<tr>
<td><strong>Information / contact</strong></td>
<td>Administration 088 75 588 29 or dd clinical microbiologist virology 71762</td>
</tr>
</tbody>
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