

Leptospirosis in Kuala Lumpur and the Comparative Evaluation of Two Rapid Commercial Diagnostic Kits Against the MAT Test for the Detection of Antibodies to *Leptospira Interrogans*

WY Sekhar, E H Soo, V Gopalakrishnan, S Devi

ABSTRACT

The aim of the study was to look into the epidemiology of serodiagnosed cases of leptospirosis at the University Hospital and compare two commercial ELISA Assays to the Microscopic Agglutination Test (MAT). Demographic data for all serodiagnosed cases for the years 1991-1997 were collected. From this data, 104 sera (n=104) were selected as samples for comparative evaluation of the commercial ELISAs (INDX Dip-S-Ticks and PanBio ELISA) to the MAT test. Thirty two (n=32) negative control sera were selected from serodiagnosed cases of other differential diagnosis of leptospira infection. The MAT test is a standard test that detects agglutination antibodies to leptospira biflexa, while the INDX Dip-S-Ticks is an ELISA dot test assaying for total anti-leptospira antibodies. The PanBio ELISA is a colorimetric assay in test well strips to detect anti-leptospira IgM. The sensitivity, specificity, and efficiency of tests were calculated at a MAT cut-off value of 1:320. Demographic data showed that leptospirosis peaks during March-May and Aug-Nov coinciding with the inter-monsoon period with more men being infected than women and more adults than children. The sensitivity, specificity, and efficiency of test for the INDX Dip-S-Ticks were 83.3%, 93.8% and 87.5% while the values for the PanBio ELISA were 54.2%, 96.9% and 71.3%. The suboptimal PanBio result could be related to the blocking effect of high IgG titres or could be related to the diagnostic MAT cut-off values used in this study. The data hence reflects a pattern of transmission that is related to "wet" occupational risk factors. The commercial assays evaluated, are easier to perform but interpretation of results should be based on level of endemicity. The INDX Dip-S-Ticks allows this flexibility and is a practical alternative to the MAT test.

Keywords: leptospira, epidemiology, diagnostic assay, ELISA, Dipsticks.

Singapore Med J 2000 Vol 41(8):370-375

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by spirochaetes, *Leptospira interrogans*. There are over 270 serovars of leptospire, which are traditionally classified into two broad groups based on pathogenicity. The pathogenic *L. interrogans*, is a collective of 210 serovas, and the non-pathogenic free living *L. biflexa* consists of 63 serovas. *Leptospira interrogans* from urine of infected animals infects its host by penetration through mucous membrane, or skin that is broken or macerated through prolonged immersion in water. Inoculation can occur directly, through direct contact with animals/ livestock or indirectly by contact with contaminated soil or fresh water. Upon inoculation it spreads throughout the body and crosses the blood brain barrier. After an incubation period of 2-20 days, it causes two clinical syndromes – Anicteric and Icteric Leptospirosis. Host and organ preference have been noted with certain serovars such as *canicola* with dogs, *pomona* with swine, *ballum* with mice and *icterohaemorrhagiae* with rats⁽¹⁾. In organ involvement *L. icterohaemorrhagiae*, the most virulent strain have been observed to cause renal and liver failure and serotype *grippotyphosa* or *canicola* are frequently associated with meningeal forms⁽²⁾.

An antibody prevalence study in rural Malaysia (1960-1961) have shown leptospirosis to be endemic with an antibody prevalence ratio of 11.8%. High incidence is seen particularly in oil palm estate workers and forest dwellers (28-29%) but clinical features are usually mild and does not require hospitalisation⁽³⁾. Epidemiological studies have also noted leptospirosis to be more prevalent during wet season which allows for an increase in exposure by walking barefooted on

Department of
Medical
Microbiology
Faculty of Medicine
University Malaya
50603 Kuala Lumpur
Malaysia

W Y Sekhar,
MBBS hons
Research Officer

E H Soo, MLT
Diploma
Research Officer

V Gopalakrishnan, PhD
Research Officer

S Devi, PhD
Associate Professor

Correspondence to:
Dr Shamala Devi
Email: shamala@
medicine.med.um.edu.my

puddles. Occupational risk factors include plantation workers, sewer workers, loggers, vets, abattoir workers and military personnel^(4,5). However, transmission through recreational activities involving contact with water such as swimming, canoeing and river activities have become more significant in developed economies^(6,7,8).

Leptospira are fastidious organisms requiring special culture media with long chain fatty alcohols and pyrimidines and take 4-6 weeks to grow. Serology is usually only positive by the second week of illness. Diagnosis therefore have to be based on a high index of suspicion with a history of contact with animals and occupational or recreational activities as well as clinical and laboratory parameters. Early detection of leptospira in urine and CSF by PCR diagnosis have been found to be promising but this has not been applied to service laboratories here^(9,10,11).

At the University Hospital diagnosis is made by microscopic agglutination test (MAT) for the detection of agglutination antibodies on serum and CSF, and cultures of CSF, serum, and urine. The MAT test uses the broadly reacting *L. biflexa (patoc 1)* cultured in Korthoff's media. It is cheap to perform but technically difficult and requires the maintenance of live organisms. Cultures on Korthoff's media is technically complex requiring precautions to prevent death of leptospira or overgrowth of other pathogens. The current study aims to compare the MAT test with 2 other alternative serological ELISA methods, the Leptospira IgM ELISA Test (PanBio, antigen type not specified) and the INDX Dip-S-Ticks (Integrated Diagnostics, Inc.) for the detection of total Ig to *L. biflexa (serovar patoc 1)*.

MATERIALS AND METHOD

Patients' sera (n=104)

The study was carried out at the University Hospital, Kuala Lumpur. Serological data collected from requested MAT test for the years 1991-1998 were screened for positive cases. Demographic data including age, sex, and race were recorded. From these samples, seventy-two sera were selected for comparative evaluation with the PanBio ELISA Test and the INDX ELISA dot Dip-S-Ticks test. Single serum samples (n=38) accounted for 38 sera collected during the acute phase of leptospirosis, 3-14 days after the onset of symptoms. The remaining 34 samples were paired samples (n=34) taken during admission and again on discharge.

Thirty-two negative controls included, sera from normal healthy population (n=6) and patients with viral hepatitis (n=10), dengue haemorrhagic fever (n=4) or diagnosed infective pyrexia of unknown origin (PUO) (n=12) which are other differential diagnosis of leptospira infection.

MAT

The MAT test uses *L. biflexa (patoc 1)* cultured in Korthoff's media to detect agglutination antibodies from patient sera. Patient sera is screened at a dilution of 1:80 in normal saline. 100µl of both sera and 8-14 days old culture of *L. biflexa* is incubated for 3 hours at room temperature. After incubation, a drop (approximately 6µl) of the above is placed on a slide and examined for agglutination under a dark-field microscope. Cultures showing agglutination at this dilution is further tested at serial two fold dilution up to 1:5,160. A MAT test is considered borderline at titres of 160-320 and positive at titres of $\geq 1:320$ for single samples.

The ELISAs were carried out as per the manufacturer's instructions as briefly described below.

Dip-S-Ticks Assay

The INDX Leptospira Dip-S-Ticks assay utilizes an enzyme-linked immunoassay (EIA) dot technique for the detection of antibodies. Briefly the antigens were dispensed as discrete dots onto a solid membrane. After adding specimen to a reaction cuvette, an assay strip is inserted, allowing patient antibodies reactive with the test antigens to bind to the strip's solid support membrane. In the second stage, the reaction is enhanced by removal of nonspecifically bound materials. During the third stage, alkaline phosphatase conjugated anti-human antibodies are allowed to react to bound patient antibodies. Finally the strip is transferred to an enzyme substrate reagent, which reacts with bound alkaline phosphatase to produce an easily distinct spot.

This assay is performed in a waterbath at 50°C: and takes approximately an hour to perform. We assayed for total Ig against leptospirosis but when assaying for IgM only, an additional reagent proSorb G (goat anti-human IgG absorbent) can be added to the first reaction

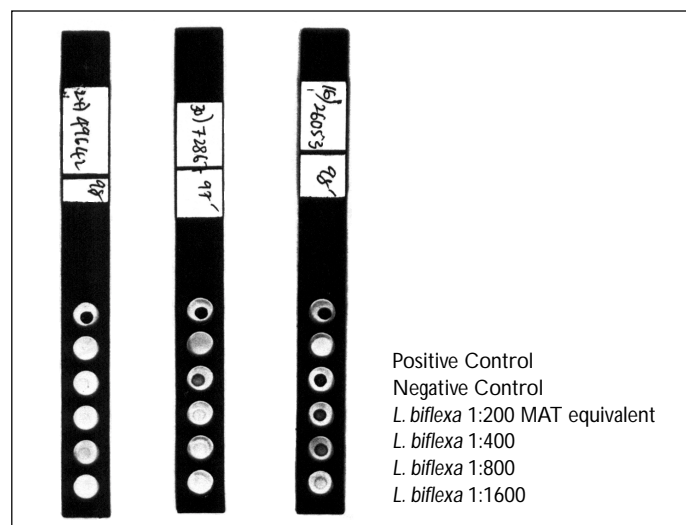


Fig. 1 Leptospira INDX Dip-S-Ticks showing (a) no reactive antibodies, (b) reactive antibodies at $> 1:400$ and (c) reactive antibodies at $> 1:1600$

cuvette and the remaining assay continues according to the same protocol as for the total Ig assay. The dip-S-Ticks contain 6 windows, the first two being the positive and negative control and the remaining four windows are the *L. biflexa* antigen at serial double dilution (Fig. 1). At the specified serum dilution, the first well represents a $\geq 1:200$ dilution. The results are interpreted based on the number or level of reactive dots and equated to the corresponding titre dilution⁽¹²⁾.

PanBio Leptospira IgM test

The PanBio kit utilises a direct ELISA format. Briefly polystyrene microwell test strips are coated with *Leptospira* antigen. Diluted serum is then added and allowed to incubate for 20 minutes at room temperature. Residual serum is then removed by washing and peroxidase conjugated anti-human IgM is added. The microwells are washed and a colourless substrate system, tetramethylbenzidine/ hydrogen peroxidase (TMB/ H_2O_2) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. The result is read with a dual wavelength spectrophotometer at 450nm and a background of 650nm. The colour intensity is directly related to the concentration of *Leptospira* IgM antibodies in the test sample.

Each set of tests is run with a positive control, negative control and cut-off calibrator in duplicate. The test is valid when the absorbance readings of the above meet the specifications of the PanBio instructions. The

results are interpreted based on PanBio Units derived from the equation:

- PanBio Units = $10 \times (\text{Absorbance of sample} / \text{mean absorbance of cutoff})$
- PanBio units of <10 gives a negative result interpreted as no evidence of recent infection,
- PanBio units of 10-20 is a low positive result and may suggest a recent infection and
- PanBio units of >20 is a positive result suggestive of a recent or current infection⁽¹³⁾.

Data Analysis

Sensitivity, specificity, positive predictive values (PPV), negative predictive value (NPV), and Efficiency of Test were calculated based on MAT cutoff of ≥ 320 dilution, using standard equations:

- % sensitivity = $\text{true positives} / (\text{true positives} + \text{false negative}) \times 100$,
- % specificity = $\text{true negatives} / (\text{false positive} + \text{true negative}) \times 100$,
- PPV = $\text{true positive} / \text{all positive test}$
- NPV = $\text{true negative} / \text{all negative test}$
- Efficiency of test = $(\text{true positive} + \text{true negative}) / \text{total samples}$

RESULTS

Demographic data

From 1991 to 1998 there has been an increase in requests for the MAT test from 50-100 requests per year to 282 requests for 1998. There is also a proportionate increase in the number of cases diagnosed, giving a the MAT test a positive yield of approximately 8%. The serological incidence detected at the University Hospital is not high, with 5-36 cases diagnosed annually. Demographic data collected from these samples shows that leptospirosis occurs in two peaks in urban Malaysia, the first peak during March-May and the second peak during Aug-Nov (Fig. 2). Although limited, our data shows that more men are infected than women with a male to female ratio of 2:1 and more adults are affected than children with no specific peak in any age groups. Race distribution shows a higher occurrence of leptospirosis in Malays (Fig. 3a & 3b)

Diagnostic Assays

The positive and negative controls in all Dip-S-Ticks were appropriate, indicating that all sera were tested correctly. Table I shows the comparative result of the three diagnostic assays. The MAT assay was considered positive at $\geq 1:320$. We had considered the Dip-S-Ticks assay as positive for leptospira infection at a higher titre of $\geq 1:400$ as it is assaying for total immunoglobulins rather than agglutination antibodies. Based on these values the Dip-S-Ticks had tested positive for 40 out of

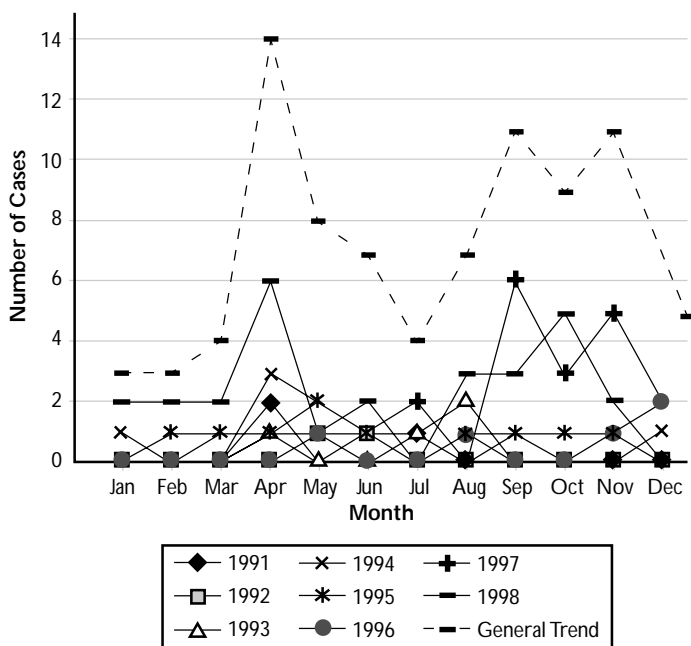


Fig. 2 The incidence of *Leptospira* MAT positive test for years 1991-1998.

48 MAT positive sera. Six out of eight of the false negative sera had a MAT titre of $\geq 1:640$. There were two false positives from the MAT negative ($< 1:80$) sera, these sera were from serologically diagnosed Hepatitis A and B samples and had stained positive at Dip-S-Ticks titres of 1:400 and 1:800 respectively. From these results, the sensitivity and specificity of the Dip-S-Ticks were calculated to be 83.3% and 93.8% respectively, the PPV is 95.2%, NPV is 79% and the efficiency of test is 87.5% at cut-off MAT titres of $\geq 1:320$.

The controls and cut-off calibrators for the PanBio ELISA assay were valid indicating that the ELISA were performed correctly. The PanBio ELISA were considered positive at PanBio units of ≥ 10 . Using this assay, a significant number of MAT positive test at lower agglutination titres were PanBio negative and only 26 out of 48 MAT positive cases were identified. The remaining 22 false negative sera were predominantly at MAT titres of $\geq 1:640$. However, at a higher MAT titre of $\geq 1:1280$, PanBio was able to pick up most of the MAT positive cases. There was 1 false positive serum from the Scrub typhus (*Orientia tsutsugamushi*) sera samples which tested positive at a PanBio unit of 13.12. The sensitivity, specificity, PPV, NPV and efficiency of test of the PanBio ELISA were calculated to be 54.2%, 96.9%, 96.3%, 58.5% and 71.3% at MAT cut-off titres of $\geq 1:320$.

DISCUSSION

Although leptospirosis is endemic in rural Malaysia, the number of requests and diagnosed cases at the University Hospital located in an urban setting, indicates that leptospirosis is either under-diagnosed or the transmission cycle in this setting is limited. The demographic data obtained from seropositive cases diagnosed at the hospital is hence limited and insufficient for conclusion although it concedes with frequently observed patterns of transmission related to "wet" occupational risk factors^(14,15).

Leptospirosis is a systemic infection with the primary lesion being endothelial cell membrane defects of small vessels leading to haemorrhage, ischaemia and secondary organ pathology. Clinically, it differs from other vasculitic or haemorrhagic infection through distinguishing clinical and laboratory features. Anicteric leptospirosis which accounts for the majority of cases (85-90%) is usually biphasic in nature with a septicaemic stage that lasts for 4-7 days, then a quiescent period of 1-3 days followed by an immune stage that can last from 4 to 30 days. The onset of the septicaemic stage is abrupt with high remitting fever, headache, myalgia, abdominal pain, nausea and vomiting, typically non-purulent conjunctival suffusion, rash, and lymphoid organ enlargement. *Leptospira* can be isolated from the

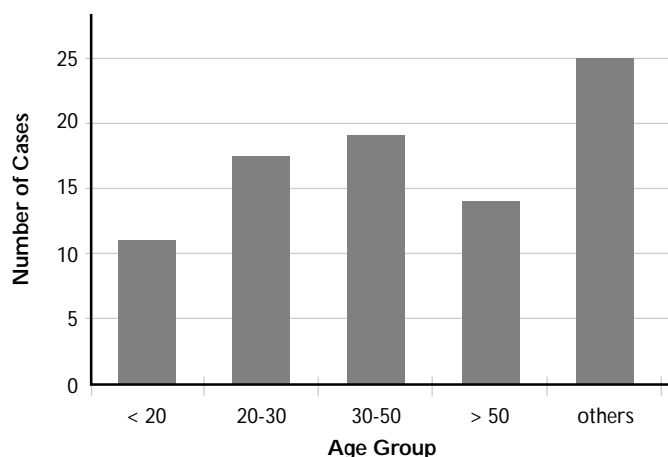


Fig. 3a Age group incidence of Leptospirosis diagnosed at the University Hospital for years 1991-1998.

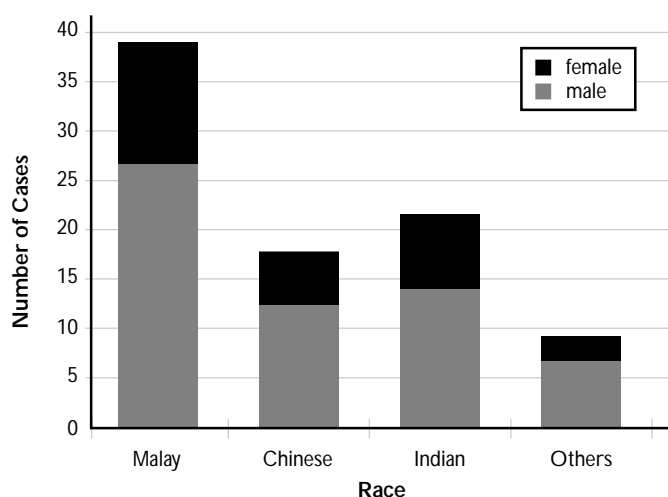


Fig. 3b Gender and race distribution of Leptospirosis diagnosed at the University Hospital for years 1991-1998.

Table I. Comparative evaluation of MAT, INDX Dip-S-Ticks and PanBio ELISA at MAT cutoff of 1:320

Diagnostic Tests	MAT positive MAT $\geq 1:320$	MAT negative (Negative Control)	Total
INDX Dip-S-Ticks positive	40	2	42
INDX Dip-S-Ticks negative	8	30	38
PanBio ELISA positive	26	1	27
PanBio ELISA negative	22	31	53
Number of sera	48	32	80

blood and CSF during this phase. The immune stage follows, thought to be due to the onset of an immune response hence appearance of IgM. Within 1-2 days, leptospirae are cleared from the CSF and blood giving aseptic meningitis and milder symptoms. Leptospirae

remains in the renal tubules and are excreted in the urine in 95% of cases. This can persist for 4-6 weeks hence urine cultures and serology is of diagnostic value during this phase^(5,19).

Icteric leptospirosis (10-15%) or Weil's disease has more severe manifestations with symptomatic hepatic and renal failure, and bleeding diathesis associated with a 10%-50% mortality rate⁽¹⁵⁾. There is usually no demarcation between the two phases and organ failure can occur as early as the third day of illness. Jaundice as its name implies is a prognostic factor and is related with liver function failure at a subcellular level, thought to be enzyme or toxin mediated. Histochemical studies shows lime hepatocellular necrosis hence the rise in transaminases are relatively small. Severe vasculitis amplified by impaired liver production of prothrombin, albumin and globulin causes haemorrhagic and circulatory manifestations. Peri-myocarditis with ECG abnormalities can further contribute to this hypoperfusion. Anaemia, thrombocytopaenia, leucocytosis with neutrophilia are common haematological laboratory findings. Renal pathology secondary to ischaemia, results in azotemia, oliguria and anuria. Rhabdomyolysis especially of the gastrocnemius muscle, hence tender calves, releases creatinine kinase and myoglobin which have been suggested to increase the severity of renal failure. Pulmonary involvement are usually haemorrhagic manifestations that are bilateral, interstitial and peripheral causing cough, dyspnoea, haemoptosis/ frank haemorrhage and respiratory failure. Poor prognostic features associated with higher mortality includes jaundice, anuria and rhabdomyolysis (50%)^(5,19).

Antibiotic treatment with penicillin, amoxicillin, ampicillin, doxycycline, and tetracycline have been shown to decrease the duration of the illness and if commenced within the first 3 days, will decrease the severity but mortality rate have not been shown to be affected^(14,15). Jarisch Herxheimer reaction is rare but has occurred, and is thought to be due to sudden release of toxins and can be fatal. Treatment is otherwise supportive and requires close observation. Early diagnosis is hence important for the treatment of patients and also allows for appropriate preventative measures by health authorities which includes surface disinfection, quarantine of animal farms, rodent control, regulations on appropriate protective occupational clothing, education, prophylactic doxycycline and animal and human vaccination.

When comparing the practical aspects of the three tests, the ELISAs are easier to perform, taking approximately an hour to complete, and is simpler to interpret. The INDX Dip-S-Ticks is a self-contained test with each Dip-S-Tick containing the controls and

antigen wells. It requires a waterbath or a heat block beyond the basic laboratory apparatus. The PanBio microwell test strips contain 8 wells per strip of which 4 wells are used for control in every run. It is therefore better to run this test when there are many samples to avoid wastage of test wells. It requires a microplate washing system and an ELISA reader. The MAT test requires a tissue culture system to maintain fresh leptospira cultures and a dark-field microscope to read the agglutinated cultures on slide. It is only done against one serovar and is a difficult test to standardise especially when dealing with live organisms. Interpretation is also based on a subjective appraisal of the presence of agglutination.

The analytical evaluation of the two commercial ELISA kits against the MAT positive sera shows that the INDX Dip-S-Ticks which uses the same antigen, *Leptospira biflexa (patoc 1)* has a sensitivity of 83.3%, specificity of 93.8%, PPV of 95.2%, NPV of 79% and efficiency of test at 87.5% at a cut-off MAT titre of $\geq 1:320$. Cross-reactivity occurred with two sera from viral hepatitis. The PanBio IgM ELISA uses a non-specified leptospira antigen which have been demonstrated to detect a number of *L. interrogans* serovars. This test, when performed and interpreted according to the manual instructions resulted in a compromised sensitivity of 54.2% compared to a high specificity of 96.9%, the NPV is 58.5%, the PPV is 96.3% and the efficiency of test is 71.3%. However at a higher MAT titre 1:1280 the PanBio ELISA was able to pick up almost all the positive sera. Cross reactivity occurred with one serum from scrub typhus. The numerous false negative results could be related to the type and quantity of antigen used as well as the MAT cut-off value of 1:320. However we should note that the PanBio kit is measuring only IgM titres while both the other two are measuring total antibodies. It is also well known that high IgG titres can block binding of IgM resulting in a false negative value. This evaluation differs significantly with previously done studies on both kits giving sensitivities of 100% when the cut-off MAT titre were 1:800^(12,17). The incidence of leptospirosis differs regionally, and appears to be more prevalent in a rural setting where man transgresses into the zoonotic cycle of transmission. A MAT titre of $\geq 1:800$ may therefore only be diagnostically significant in an endemic region where there is continual re-exposure. In a non endemic region a lower titre is significant. Our study at a MAT cut off of $\geq 1:320$ is based on the University Hospital's diagnostic criteria as generally a MAT of $\geq 1:400$ is considered positive in non-endemic areas^(16,17,18). Commercial kits therefore should tailor their kits to allow flexibility of interpretation based on endemicity.

In conclusion, in view of the re-emerging zoonosis, the prompt diagnosis of leptospirosis is essential for both patient care and efficient implementation of public health measures. It is therefore important to have an efficient diagnostic test that is rapid, accessible and practical to general physicians. In our evaluation of a limited number of sera (n=104), the INDX Dip-S-Ticks appears to be a practical alternative but our evaluation of PanBio is suboptimal and could be related to our current MAT positive selection criteria for leptospira sera and to the blocking effect of high IgG titres. PCR diagnosis of urine, CSF and blood samples could further improve diagnosis and could be established in specialist centres.

ACKNOWLEDGEMENTS

We thank PanBio for providing the *Leptospira* IgM ELISA Test and Integrated Diagnostics, Inc for providing the INDX leptospira Dip-S-Ticks. We also thank R A Vinsent for his assistance in the ELISA Assays.

REFERENCES

1. Faine S. Leptospirosis. Infectious Disease, Fifth Edition. 1994. Editors Hoeprich, Jordan Ronald: Chapter 65, 619-25.
2. Dupont, Dupont-Perdrizet D, Perie JL, Zehner-Hansen S, Jarrige B, J.B.Daijardin. Leptospirosis: Prognostic factors associated with mortality. CID 1997; 25:720-4.
3. Tan SK. Leptospirosis in Rural West Malaysia. Med J of Malaysia 1970; 4:261-6.
4. Ng TP. Epidemiology of Leptospirosis in Singapore. Infectious Diseases 1981; 319-24.
5. Farr. Leptospirosis. State-of-the-Art Clinical Article. Clin Inf Disease 1995; 21:1-8.
6. Murhekar, Sugunan AP, Vijayachari P, Sharma S, Sehgal SC. Risk factor in the transmission of leptospiral infection. Indian J Med Res 1998; 107:218-23.
7. Outbreak of acute febrile illness among athletes participating in triathlons-Wisconsin and Illinois, 1998. JAMA 1998; 280(17):1473-5.
8. Outbreak of leptospirosis among white-water rafters-Costa Rica, 1996. JAMA 1997; 278(10):808-9.
9. Gravekamp, Kemp VD, Franzen M, Carrington D, Schoone GJ, G. Van Eys G, et al. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiology 1993; 139:1691-700.
10. Bal, Gravekamp C, Hartskeerl R, Meza-Brewster JD, Korver H, Terpstra W. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Micro 1994; 32(8):1894-8.
11. Romero, Billerbeck A, Lando V, Camargo E, Souza C, Yasuda P. Detection of leptospira DNA in patients with aseptic meningitis by PCR. J Clin Micro 1998; 36(5):1453-5.
12. INOX Dip-S-Ticks. Leptospira. Cat # 5065-01-50. Rev 6 1997.
13. PanBio. Leptospira IgM ELISA Test. Cat No. LPM-200. Rev 1998.
14. Kendell. Leptospirosis update. BMJ 1991; 302:301-2.
15. George Watt. Leptospirosis. Current Science 1992 ISSN 0951-7375: 659-63.
16. Silva D, Nakamura P, Camargo E, Batista L, Vaz J, Romero E, et al. Immunodiagnosis of human leptospirosis by Dot-ELISA for the detection of IgM, IgG and IgA antibodies. Am Soc of Trop Med and Hygiene 1997; 56(6):650-5.
17. Winslow, Merry D, Pire M, Devine P. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J Clin Micro 1997; 35(8):1938-42.
18. Watt L, Alquila L, Padre L, Tuazan M, Lauglalin L. The rapid diagnosis of leptospirosis: A prospective comparison of the dot enzyme-linked immunosorbent assay and the genus-specific microscopic agglutination test at different stages of illness. J Inf Dis 1988; 157(4):840-2.
19. Kaufmann, Weyant RS. Leptospiraceae. Bacteriology 19, Chapter 50: 621-2.