An evaluation of dark field microscopy, culture and commercial serological kits in the diagnosis of leptospirosis

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Abstract

Context: This study was conducted to analyze the clinical utility of various leptospira diagnostic modalities. Aims: To evaluate the role of dark field microscopy (DFM), culture, immunochromatography (IgM Leptocheck), IgM enzyme-linked immunosorbent assay (IgM ELISA), macroscopic slide agglutination test (MSAT) and microscopic agglutination test (MAT) in diagnosing leptospirosis in febrile patients. Settings and Design: Descriptive study conducted in a tertiary care hospital from January 2011 to April 2012. Subjects and Methods: Blood, urine and paired sera from 100 patients with clinical suspicion of leptospirosis (study group) were collected and subjected to DFM, culture, IgM Leptocheck, IgM ELISA and MSAT. Fifty randomly selected sera from febrile patients tested positive for infections other than leptospirosis (control sera) were also subjected to the aforementioned serological assays. All the leptospira seropositive samples were subjected to MAT. Statistical Analysis Used: Positive predictive values (PPV) and coefficient of agreement (kappa). Results: None of the clinical samples showed positivity by DFM. Leptospira inadai was isolated from a urine sample. The seropositivity of IgM Leptocheck, IgM ELISA and MSAT was 16%, 46% and 47%, respectively. The PPV of these assays was 14.3%, 8.7% and 6.5%, respectively. Poor agreement was obtained among these assays. Only four study group leptospira seropositive samples were confirmed by MAT with Australis being the predominant serovar. None of the leptospira-positive control sera were confirmed by MAT. Conclusions: DFM and culture have limited utility in diagnosing leptospirosis with serology being the mainstay. The present study shows discordant results with the commercially available serological kits. Further studies should be done to evaluate the various diagnostic modalities.

Key words: Culture, dark field microscopy, leptospirosis, serology

Introduction

Leptospirosis is considered as the most widespread zoonosis in the world with higher incidence in the tropics (most of which are constituted by developing countries) than temperate regions.[1-3] A survey conducted by International Leptospirosis Society (ILS) in various countries showed that thousands of leptospirosis cases occur annually world-wide. However, this could only be an underestimate of the actual incidence.[1] Leptospirosis bears clinical resemblance with several infectious diseases.[4] The most common and severe clinical syndromes are anicteric and icteric leptospirosis, respectively.[5] Complications include acute respiratory failure, myocarditis, menigitis, renal failure, uveitis and death.[1,5] Since the mainstay of diagnosis is microbiological,[6-9] therefore, this study aimed to evaluate role of dark field microscopy (DFM), culture, immunochromatography (IgM Leptocheck), macroscopic slide agglutination test (MSAT), IgM enzyme-linked immunosorbent assay (IgM ELISA) and microscopic agglutination test (MAT) in diagnosing leptospirosis in febrile patients.

Subjects and Methods

A descriptive study was conducted in a tertiary care hospital from January 2011 to April 2012. The study group consisted of 100 patients attending outpatient departments (OPDs) or admitted in Paediatric and Medicine wards. The inclusion criteria for selecting these patients were: (a) Patients with undiagnosed fever of ≤2 weeks in duration. (b) Patients with fever and jaundice. (c) Patients with fever with other clinical manifestations such as headache, conjunctival suffusion,
myalgia, rashes, oliguria/anuria and features suggestive of meningitis. (d) Patients with aforementioned clinical manifestations with occupational history of farming/field work, mining, sewage cleaning and those indulging in various recreational activities like swimming and hunting. However, patients on antibiotic (crystalline penicillin and tetracyclins, etc.) treatment and those less than 1 year of age were excluded from the study. Relevant clinical and epidemiological data was obtained as per the proforma formulated. Blood, urine and paired serum samples (obtained 1 week apart on seventh and fourteenth day of fever, respectively) were collected using aseptic precautions after obtaining an informed consent.

Fifty randomly selected unpaired serum samples, which were tested positive for infections other than leptospirosis like enteric fever, Hepatitis B, dengue and human immunodeficiency virus (HIV) (by widal, HBsAg ELISA, dengue IgM immunochromatographic card test and HIV ELISA, respectively) were obtained from febrile patients and referred to as control sera. These sera were also subjected to the aforementioned leptospira serological assays. We intended to use these samples as controls along with the in-built controls which were provided with all the commercially available leptospira serological kits used in our study.

Institutional ethical approval was obtained and the following samples were collected:

**Blood**

Five ml of intravenous blood samples were collected aseptically from patients between seventh to tenth day of fever in sterile vacutainers coated with Ethylene diamine tetra acetic acid (EDTA) for DFM and culture.

**Urine**

Freshly voided mid-stream urine samples were collected from patients between tenth to fourteenth day of fever in sterile containers containing equal amount of Phosphate buffered saline (PBS) with pH 7.2.

**Sera**

Two millilitre of intravenous blood samples were collected aseptically in plain vacutainers on two occasions (obtained one week apart on seventh and fourteenth day of fever, respectively) for serological assays like IgM Leptocheck, IgM ELISA, MSAT and MAT. Haemolysed, clotted, contaminated, viscous/turbid specimens were rejected. Serum samples were stored at −20°C, if not processed immediately. Repeated freeze-thawing was avoided. No preservatives were used for storing serum samples.

The following procedures were carried out on the samples collected.

**DFM**

**Blood**

Blood sample (treated with an anticoagulant) was centrifuged at 1000 g for 15 minutes. Approximately 10 μl of plasma was placed on a thin microscopic slide and cover slip was placed. The wet films were examined under DFM under low and high power magnifications (×100 and ×400). If no leptospires were seen, the plasma was centrifuged at 3000–4000 g for 20 minutes. The supernatant was removed carefully and a drop of sediment was examined microscopically as above. [10]

**Urine**

A portion of freshly voided urine was centrifuged at 3000 g for 10 minutes. A drop of deposit was then placed on a thin microscopic slide and covered with a cover slip to prepare a wet film and examined under DFM under both low and high power magnifications (×100 and ×400), respectively. [10]

**Culture**

Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid medium was used. Commercially available leptospira medium base and leptospira enrichment (BD-Difco) were used to prepare the culture medium. Selective EMJH medium was prepared by adding 100 μg/ml of 5-Fluoro Uracil (Rolex Chemical Industries) to the original medium for inoculating urine samples.

**Blood**

Few drops of blood were inoculated into sterile capped plastic centrifuge tubes containing about 5 ml medium and incubated at 28–30°C for 4–6 weeks. [10, 11]

**Urine**

Urine which was diluted in PBS 7.2 was inoculated into sterile capped plastic centrifuge tubes containing selective EMJH medium. Subculture was done into EMJH medium without 5-Fluoro Uracil within 48 hours to minimize the inhibitory effect of selective agent on leptospira. This was then followed by incubating these cultures at 28–30°C for 4–6 weeks. [10, 11]

**Examination of the cultures**

The cultures were examined for signs of growth, i. e., turbidity, haze or a ring of growth (Dinger’s ring) and by using dark field illumination initially on day one, three and five, followed by seven to ten days interval up to 6 weeks. [10] When the tube inoculated with a specimen became contaminated before 6 weeks, filtration of the medium was done using a 0.22 μm membrane filter using syringe filters to remove the contaminants. The filtrate was then inoculated into a fresh medium. Lightly contaminated cultures were sub-cultured into selective EMJH medium. [12]
Reporting of the results

Cultures were incubated for 4–6 weeks before reporting them as negative. If all the tubes inoculated with a specimen became contaminated beyond retrieval before 6 weeks, it was reported as contaminants isolated in culture. All the positive cultures were reported as ‘Leptospires isolated’.

Serology

For IgM Leptocheck, IgM ELISA and MSAT kits obtained from Zephyr Biomedicals, J. Mitra and Co. Pvt. Ltd. and Bio-Rad, respectively were used according to manufacturer’s instructions. All serum samples testing positive by leptospira serological tests (study group and control sera) were sent to Regional Medical Research Centre (Indian Council of Medical Research), World Health organisation (WHO) collaborating centre for diagnosis, reference, research and training in leptospirosis, Port Blair, Andaman and Nicobar islands (India) in cold chain to be tested by MAT. Sera with titres >/=1:80 were interpreted as positive by MAT.\[^{[13]}\]

Results

Out of 100 patients who constituted study group, twenty presented with high grade fever, ten with headache, twenty with bilateral conjunctival suffusion, fifteen with meningism, thirty-one with myalgia, twenty-four with jaundice and thirty-one with signs of renal dysfunction in various combinations. [Table 1] Sixty of these patients presented during rainy seasons (May–August and October–November). Only five patients gave positive history of contact with contaminated environment. None of these patients gave positive history of animal contact. Sixty percent of study group patients were factory workers, 30% were school going children and 10% were home-bound dependents of ESI beneficiaries.

DFM

None of the blood and urine samples collected were tested positive by DFM.

Culture

Leptospires were isolated from urine of a one year old child [Figures 1 and 2] and identified as *Leptospira inadai* by polymerase chain reaction (PCR) performed at Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS, Hebbal, Bengaluru). Both acute and convalescent sera of this patient were tested positive by IgM Leptocheck, IgM ELISA and MSAT. However, this serum was tested negative by MAT.

Serology

Sixty-three study group patients gave paired sera. The serum samples (acute and/or convalescent) of 49 of these patients were tested positive for leptospirosis by IgM Leptocheck, IgM ELISA and MSAT in various combinations as shown in Table 2. IgM Leptocheck detected seropositivity in 16 (16%), IgM ELISA in 46 (46%) and MSAT in 47 (47%) patients, respectively.

All the 49 study sera testing positive by either of the three serological tests were subjected to MAT. The various serovars used in MAT were *Australis*, *Bankinang*, *Canicola*, *Grippotyphosa*, *Hebdomadis*, *Icterohaemorrhagiae*, *Pomona*, *Pyrogenes* and *Hardjo*. Only sera of four of these patients were tested positive by MAT (three of which were tested positive for serovar *Australis* and one for two serovars, i.e., *Grippotyphosa* and *Pyrogenes*). MAT detected two out of sixteen patients testing seropositive by IgM Leptocheck, four out of forty-six IgM ELISA seropositive patients and three out of forty-seven MSAT seropositive patients.

Out of fifty control sera, twenty two, twenty one, four and three tested positive for enteric fever, Hepatitis B, dengue and HIV by widal test, HBsAg ELISA, dengue IgM immunochromatographic card test and HIV ELISA, respectively. Thirty-eight of these fifty serum samples tested positive for leptospirosis by MSAT and IgM ELISA (fourteen by only MSAT; four by only IgM ELISA and twenty by MSAT and IgM ELISA taken together). None of these sera tested positive by IgM Leptocheck. All the 38 samples tested negative by MAT.

Table 3 shows the positive predictive value (PPV) of IgM Leptocheck, IgM ELISA and MSAT of study group and control sera. Coefficient of agreement (Kappa) between different serological assays for leptospirosis in study group and control sera was calculated by using statistical package of social sciences (SPSS) software and is shown in Table 4. A kappa correlation of >/=0.75 was considered a good agreement; 0.45–0.74 was a fair agreement and </=0.45 a poor agreement.

In our study, agreements between none of the serological tests performed on study group sera were found to be statistically significant. For control sera, although the kappa

| Table 1: Clinical profile of study group patients |
|-----------------------------------------------|
| **Clinical manifestations** | **No. of cases** |
| High-grade fever (>39°C)** | 20 |
| Headache | 10 |
| Bilateral conjunctival suffusion | 20 |
| Meningism | 15 |
| Myalgia (especially of calf muscles) | 31 |
| Jaundice | 24 |
| Signs of renal dysfunction (oliguria/albuminuria/raised blood urea nitrogen) | 31 |

*Table formulated as per modified Faine’s criteria.*\[^{[14]}\]**All 100 patients presented with fever out of which 20 presented with high-grade fever
correlation between IgM ELISA and MSAT was found to be low indicating poor agreement, the agreement between these assays (indicated as %) was found to be statistically significant. Some agreement was also obtained between IgM ELISA-IgM Leptocheck and MSAT-IgM Leptocheck but the statistical significance of this agreement could not be evaluated as the kappa correlation between these tests was zero indicating poor correlation.

Discussion

Leptospirosis is an emerging infectious disease with increasing incidence in both developing and developed countries. Though it is sub-clinical or mild in most cases, severe illness can sometimes end fatally. The clinical presentation is difficult to distinguish from dengue, malaria, influenza and many other diseases characterized by fever, headache and myalgia. The differential diagnosis of leptospirosis depends on the epidemiology of acute febrile illnesses in the particular area. A high index of suspicion is needed in endemic areas and leptospirosis must be considered when a patient presents with acute onset of fever, headache and 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.

In our study, PPV of IgM Leptocheck, IgM ELISA and MSAT (taking MAT as the gold standard test) were found to be extremely low (14.3%, 8.7% and 6.5%, respectively for sera obtained from study group). The reasons for this low PPV could be due to the various diagnostic pitfalls of MAT like: (a) The antibodies may not be detectable when the causative strain is not represented in the panel (of serovars used for MAT) or (b) Only a low titre is found with a serovar that antigenically resembles the absent causative serovar and (c) It is never possible to be sure that the panel is complete since, new, unidentified leptospires may cause the disease.

Also, paired sera could be obtained from only 63 out of 100 study group patients as a result of which many of the potential leptospira seropositive samples might have been missed.

Antibodies against leptospirosis usually develop within 2–12 days after the onset of illness. IgM antibody starts appearing early in the course of the disease and reaches detectable levels within one week or as early as third or fourth day of illness. They reach peak levels during third or fourth week and then decline slowly over months and then become indetectable within 6 months. In a small proportion of patients, IgM antibodies may not develop at detectable level and the first appearing antibody may be IgG. Hence, the sera from these patients may give negative results in IgM based immunoassays. In about 10% of patients, microscopic agglutinating antibodies appear in detectable levels only after about a month of illness. Hence, the sera collected from these patients during the first month of illness may give negative results in MAT.

Many studies have shown that immunochromatography (ICT), IgM ELISA and MSAT which are used for diagnosing leptospirosis have much higher PPV.[15,17,18] In most of these studies, only sera from cases of leptospirosis (confirmed by MAT) were subjected to various rapid leptospira serological tests like IgM ELISA, ICT and MSAT and validity of these tests was evaluated. However,

| Table 2: The positivity of leptospira serological tests in various combinations in study group patients |
|-----------------------------------------------|
| No. of patients tested positive by IgM Leptocheck, IgM ELISA | No. of patients tested positive by MSAT, IgM ELISA | No. of patients tested positive by IgM Leptocheck and MSAT | No. of patients tested positive by IgM Leptocheck | Total |
|-----------------------------------------------|
| 11 | 33 | 3 | 2 | 49 |

IgM Leptocheck: Immunochromatography, MSAT: Macroscopic slide agglutination test, IgM ELISA: IgM enzyme-linked immunosorbent assay

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in the present study, sera from clinically suspected cases of leptospirosis were first screened by rapid leptospira serological tests and then all the seropositive samples were subjected to MAT for confirmation of diagnosis. This also probably explains the lower PPV in our results.

Among control sera, the PPVs of MSAT and IgM ELISA were found to be 0% each. The reason for this finding could be due to the various shortcomings of serodiagnosis of leptospirosis by MAT as cited above. Although, all the controls (positive and negative) provided with commercially available leptospira serological kits gave appropriate results, but, the results obtained with the fifty control sera used in our study may be inferred as co-infection or serological cross-reactivity. This mandated the need to get thirty eight out of fifty leptospira positive control sera evaluated by MAT (serological gold standard for leptospirosis) along with forty nine out of hundred leptospira positive study group sera.

Dual infections with leptospires and other etiologic agents like Dengue virus, Human Immunodeficiency Virus, Hepatitis B and E viruses, etc., have rarely been reported. Serological cross-reactivity between leptospirosis and other infectious diseases has also been reported.[13-15] However, the results obtained are largely inconclusive about the possibility of dual infections as possible mixed infections should be confirmed by culture or detection of specific nucleic acid sequences which unfortunately could not be incorporated in the present study. Also, a grading system analogous to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) guidelines and Infectious Diseases Society of America guidelines for evaluating the strength of evidence for mixed infections should be considered.[19]

All the three serological assays (i.e. IgM Leptocheck, IgM ELISA and MSAT showed low level of concordance among themselves in detecting seropositives among study group and correctly categorizing control sera as negative (as indicated by kappa correlation and agreement statistics). Since sensitivity and specificity of these diagnostic assays could not be evaluated in the present study (due to some unforeseen circumstances), therefore, it is difficult to comment about such a finding. However, poor agreement among these diagnostic assays could possibly be due to their low seropositivity (as indicated earlier).

In the present study, sera of three out of four cases tested positive by MAT had displayed antibodies against serovar Australis. Serum sample of one patient displayed antibodies against two serovars (i.e.) Grippotyphosa and Pyrogenes. This could be due to: (1) Although, patients usually produce agglutinating antibodies against the infecting serovar, but antibodies that cross-react with other serovars are also often found. This is particularly noticeable in the early course of infection. (2) In the first few weeks of the disease, heterologous cross-reactions with other serovars may be even stronger than the homologous reaction with the infecting serovar. Occasionally, a heterologous reaction can be positive while the homologous reaction is or remains negative, a phenomenon called as paradoxical reaction. (3) Simultaneous infections from multiple serovars are possible though not as widely reported as expected.[11,20]

Also, out of the four patients tested positive by MAT, all four were also tested positive by IgM ELISA, three by MSAT and two by IgM Leptocheck. None of the 100 cases were tested positive for leptospirosis by DFM. However, DFM has not been accepted for diagnostic purposes; as it is considered insensitive and the results are non specific.[13]

Leptospires were isolated from only one patient and identified as Leptospira inadai by PCR. Although both acute and convalescent serum samples of this patient were tested positive by IgM Leptocheck, MSAT and IgM ELISA, these were tested negative by MAT due to non-inclusion of Leptospira inadai in the panel of serovars used for performing MAT.

Table 3: The positive predictive values of leptospira serological tests performed on study group and control sera

| Test                  | Positive predictive value (%) | Study group sera | Control sera |
|-----------------------|-------------------------------|-----------------|--------------|
| IgM Leptocheck        | 14.3                          |                 | *Not applicable |
| IgM ELISA             | 8.7                           |                 | 0            |
| MSAT                  | 6.5                           |                 | 0            |

IgM Leptocheck: Immunochromatography, MSAT: Macroscopic slide agglutination test, IgM ELISA: IgM enzyme-linked immunosorbent assay *The PPV of IgM Leptocheck could not be calculated as none of the control sera were tested positive by this assay

Table 4: Agreement between serological diagnostic assays for leptospirosis in study group and control sera

| Assays compared       | Agreement (%) | Kappa correlation | P value |
|-----------------------|---------------|-------------------|---------|
|                       | Study group sera | Control sera | Study group sera | Control sera | Study group sera | Control sera |
| IgM ELISA-MSAT        | 58            | 64                | 0.109   | 0.29         | 0.266   | 0.026   |
| IgM ELISA-IgM Leptocheck | 51          | 52                | 0.114   | 0.00         | 0.09    | -       |
| MSAT-IgM Leptocheck   | 43            | 32                | 0.08    | 0.00         | 0.164   | -       |

IgM Leptocheck: Immunochromatography, MSAT: Macroscopic slide agglutination test, IgM ELISA: IgM enzyme-linked immunosorbent assay, **For each assay, a case was considered positive if the acute and/or convalescent phase sera tested positive. P value was calculated to ascertain whether or not the agreement between different diagnostic assays (indicated as %) was statistically significant.
Conclusion

The diagnosis of leptospirosis (both laboratory and clinical) is an uphill task. Leptospirosis is an enigmatic disease and presents with various challenges for both clinicians and laboratory physicians. Although, the results obtained in our study may not be considered conclusive, but they probably reflect certain lacunae in the laboratory diagnosis of leptospirosis. Further studies should be done to evaluate and validate the various diagnostic modalities which would not only aid clinical diagnosis during initial phase of this disease but also help in rapid case confirmation during outbreak surveillance.

Acknowledgements

Faculty of Medicine, Employees’ state Insurance Corporation Medical College and Post Graduate Institute of Medical Science and Research, Bengaluru, Faculty of Pediatrics, Employees’ state Insurance Corporation Medical College and Post Graduate Institute of Medical Science and Research, Bengaluru, Faculty of PD_ADMAS, Hebbal, Bengaluru, Dr. P. Vijayachari, Director, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India, Dr. G. Jayalakshmi, Professor and HOD, Department of Microbiology, Madras Medical College, Chennai.

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How to cite this article: Bhatia M, Umapathy BL, Navaneeth BV. An evaluation of dark field microscopy, culture and commercial serological kits in the diagnosis of leptospirosis. Indian J Med Microbiol 2015;33:416-21.

Source of Support: Nil, Conflict of Interest: None declared.