Comparative Assessment of Sero-diagnostic Techniques, MAT, MSAT, and ELISA for the Detection of Anti-leptospiral Antibodies in Human Sera

T. Viswanathan[1*], A. Michael[2], K.R. Mani[3], and R. Gowrishankar[4]

[1]Department of Microbiology, Dr.G.R.Damodaran College of Science, Coimbatore, Tamil Nadu, India

[2]P.S.G. College of Arts and Science, Coimbatore, Tamil Nadu, India

[3]Pasteur Institute of India, Coonoor, Tamil Nadu, India

[4]Center for Disease Control and Prevention (CDC), Atlanta, GA 30333, USA.

Abstract

Sera from seventy-five patients with suspected leptospiral infection were examined for anti-leptospiral antibodies with three serodiagnostic techniques, *viz.*, microscopic agglutination test (MAT), macroscopic slide agglutination test (MSAT) and enzyme linked immunosorbant assay (ELISA). Primarily, MAT was employed to elucidate the serotypic identity of the leptospiral pathogen with the detection of serotype specific antibodies in the patient's serum. Thirty nine percent of the MAT positive sera had anti *L. autmnalis* antibodies. High degree of correlation (r = 0.97) existed between MAT and ELISA anti-leptospiral antibody titers. But, ELISA showed slightly higher seropositivity (84%) compared to MSAT (81%) and MAT (79%). Even though MSAT is a subjective technique, on the basis of its cost and simple procedure, it can be used as a base line or presumptive serodiagnostic technique to detect the presence of anti-leptospiral antibodies in a patient's serum.

Keywords : ELISA, leptospirosis, MAT, MSAT, serodiagnosis

INTRODUCTION

Leptospirosis, a zoonosis with worldwide distribution is an acute febrile illness caused by spirochetes of the genus Leptospira. Adolf Weil first reported this disease in 1888 among the agricultural workers with febrile illness. Human beings are the accidental hosts and become infected through direct or indirect contact with urine or tissues from infected animals (eg. livestock). Leptospira enters the human body through cuts or abrasions in skin, or mucosal membranes (Turner, 1968). The severity of this acute febrile illness varies from mild to rapid fatality, and the wide spectrum of symptoms makes the clinical diagnosis unreliable. Therefore, laboratory diagnosis is vital and till now serology has been the cornerstone in the differential laboratory diagnosis for this disease. The agglutinating anti-leptospiral antibody in serum has been studied extensively and used in leptospiral diagnosis (Schuffner and Mochtar, 1927). The microscopic agglutination test (MAT) remains the definitive diagnostic test (Turner, 1968). The "gold standard" serodiagnostic method, MAT is serovar specific, but requirement of live antigens that necessitates the maintenance of leptospiral cultures limits its use (Gupta *et al.*, 2004). Enzyme linked immunosorbent assay (ELISA) is being used to differentially diagnose the recent or chronic status of the disease with the detection of anti-leptospiral IgG or IgM, respectively. Macroscopic slide agglutination test (MSAT) was also recommended for screening serum samples as routine laboratory diagnosis for the detection of anti-leptospiral antibodies especially IgM (Brein and Weber, 1984). In order to ascertain a suitable serodiagnostic technique for diagnosis of leptospirosis, this study compares the efficiency of these 3 techniques to detect anti-leptospiral antibodies in a patient's serum.

MATERIALS AND METHODS

Strains used in this study: The reference leptospiral strains that belong to the serogroups of *Leptospira interrogans viz.*, *australis* (strain Jez Bratislava), *autumnalis* (Akiyami.A), *canicola* (H. Uterecht IV), *grippotyphosa* (Moskva V), *icterohaemorrhagiae* (RGA), and saprophytic *Semaranga* (*pattoc* 1) were obtained from Leptospira Reference Laboratory, Brisbane, Australia. All leptospiral strains were maintained with periodic sub-culturing in Ellinghausen, McCullough, Johnson and Harris (EMJH) semisolid medium (Hi–Media, Mumbai, India) supplemented with bovine serum albumin (Hi–Media, Mumbai, India) and vitamins, as recommended (Ratnam, 1994).

Blood Samples: The criterion intended for clinical diagnosis and sample collection for leptospirosis, were acute onset of fever, nausea, vomiting, headache, myalgia, jaundice, conjunctival suffusion, spleenomegaly, and renal failure. Blood samples (n = 75) were collected aseptically from August to October 2006, during the first weak of onset of symptoms (2-7 days) by the physicians in the private

*Corresponding Author
email: ***vishuwin@yahoo.co.in***

hospitals located at eastern part of Coimbatore, South India from male and female patients who convince the above stipulations. Second set of sera samples were collected 7 to 21 days after the collection of first sample during their follow up visit. Serum was separated and stored at -20°C until use for further laboratory diagnostic study.

Detection of anti-leptospiral antibodies in serum samples

***Microscopic agglutination test (MAT)*:** Strains of leptospira used as antigen were maintained in EMJH broth, incubated at room temperature for 7 days, and examined under dark field illumination. Suitable dilutions of bacterial suspension were made with phosphate buffered saline (PBS). MAT was performed adopting standard procedure (Sulzer and Jones, 1978) with the live antigenic preparations of 6 serogroups of *Leptospira interrogans* based on preceding studies (Ratnam *et al*., 1987 and Natarajaseenivasan *et al*., 2004). Two fold dilutions (1:40, 1:80, 1:160…1:10240) of serum sample were made with 1% PBS (pH 7.2) in 96 well micro titer plates. Since, the serological status for leptospirosis in Coimbatore is not comprehensible, all the serum samples were primarily screened at 1:40 dilutions and those that demonstrated more than 50% agglutination were considered as positive. Positive serum samples were subjected to quantitative MAT to determine the titer against that particular strain of leptospira (Levett, 2003).

***Enzyme linked immunosorbent assay (ELISA)*:** IgM ELISA was performed with in-house prepared ELISA micro titer plates. Plates were coated with pooled, heat inactivated whole cell antigen extracts prepared from *autumnalis* and *patoc* as described previously (Trepstra *et al.,* 1985). A serial two fold dilutions (1:40, 1:80, 1:160…1:10240) of patient's serum sample were made with phosphate buffered saline with tween (PBST) in 96 well micro titer plates and incubated for 1h. The plates were then washed with PBST and incubated with 100 µl of 1:2000 diluted peroxidase-labelled anti human IgM conjugate (Horse radish peroxidase; Sigma, St. Louis, MO) for 1h at 30°C. The plates were washed thoroughly and 100 µl of substrate (One OPD, 2HCl, tablet in 10 ml of 0.05M of phosphate citrate buffer, pH 5.0) was added to each well and were incubated in dark for 30 min. After incubation, the reaction was stopped with 50 µl stopping solution (sulfuric acid) and read at 492 nm in an ELISA reader. Samples showing antibody titer of 1:80 and above are considered to be positive.

***Macroscopic slide agglutination test (MSAT)*:** Pooled, heat inactivated whole cell antigen extracts for MSAT was prepared from *autumnalis* and *patoc* adopting the technique described earlier (Faine, 1982 and Brandão *et al.,* 1998). Briefly, the antigen suspension (55 µl) was mixed with 10 µl of undiluted serum placed on a glass slide divided into four squares (2.5 by 2.5 cm) along with positive and negative control sera and incubated in an orbital shaker (REMI, India) at 120 rpm for 4 to 6 min. at room temperature. The slide was examined under direct light against dark background for agglutination.

Statistical analysis: MAT and ELISA titers were subjected to linear regression analysis. Linear regression analysis of the Log2 transformed MAT and ELISA titers were used for determining r-values and slopes using Microsoft Excel, version 2002.

RESULTS

MAT: Serum samples from 75 patients with symptoms of leptospirosis were tested for the presence of anti-leptospiral antibodies with 3 different serodiagnostic techniques. MAT with the strains of live antigens of 6 serogroups of *Leptospira interrogans viz*., *australis*, *autumnalis*, *canicola*, *grippotyphosa*, *icterohaemorrhagiae*, and saprophytic *patoc* indicated higher incidence of *autumnalis* in Patients as 39% (23/59) of MAT positive sera reacted with the antigenic preparation of this serotype (Figure 1). Due to this, we used *autumnalis* and *patoc* as antigens in ELISA and MSAT methods. Even though 2 of 59 MAT positive serum samples had very high titer (1:10240), most of the samples had moderate (1:160) to low (1:40) titer values (r = 0.65; slope = 0.77; Figure 2). Out of 7 patients who had paired sera samples one had shown four fold rises in antibody titer and none of the other samples showed any signs of seroconversion. One sample demonstrated antibody titer in 1:320 dilutions. Other samples showed antibody titer of 1:160 in both the samples except for the two samples that were negative in both the samples. One of the MAT negative samples was picked up by ELISA with an antibody titer of 1:160.

ELISA: ELISA showed higher seropositivity compared to MAT (63/75; 84%). Higher antibody titers were obtained even in samples that had moderate titer in MAT (r = 0.52; slope = 0.73; Figure 3). The percentage of seropositivity was found to be maximum in 1:160 dilutions (22.2%). Highest titer (1:10240) for anti-leptospiral antibodies was empirical in 2 serum samples. All the 14 paired serum samples except one were found positive by ELISA. One of the paired samples was negative both in MAT and ELISA.

MSAT: MSAT exhibited positive agglutination in 61/75 (81%) of serum samples. Among these, 53% (n = 32) exhibited extremely strong MSAT reaction. The paired sera sample that failed to reveal antibody titer in MAT and ELISA was established to be negative in MSAT.

Comparison of MAT and ELISA: Two quantitative serodiagnostic techniques namely, MAT and ELISA were compared for detecting anti leptospiral antibodies

(Figure 4). Inspite of slight variation in titers of individual serum samples, both the techniques showed high level of correlation (r = 0.97; slope = 0.75) in anti leptospiral antibody detection in patient sera. Among the 3 serodiagnostic techniques, ELISA yielded more seropositivity with higher individual titers (Figure 5). The proportional analysis revealed that ELISA and MSAT showed percentage of sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive value (NPV) to an extent of 96.6/62.5/90.5/83.3% and 94.9/68.8/88.9/78.6%, respectively.

DISCUSSION

Leptospirosis popularly known as "Weils" disease was named after the German physician who recorded the etiology of the disease with a spirochete in 1886 (Subramanian and Rajendran, 1993). It is emerging as one of the most problematic infectious diseases, exemplified by large number of outbreaks (Trevejo *et al.,* 1998). Leptospirosis, though widespread and potentially lethal, often goes un-diagnosed (Wagenaar *et al.,* 2004). Most symptoms overlap with bacterial and viral diseases that often delay the diagnosis of leptospiral infection. The complexity of this is further magnified with the pathophysiology of the disease since this spirochete has a tendency of lodging itself into tissues away from circulation resulting in an abrupt drop in humoral immune response. This sudden latency not only results in protracted illness, but also throws the conventional serological diagnostic techniques out of gear. Hence, it is imperative to develop a comprehensive diagnostic tool for direct or indirect detection of leptospiral infection. Three serodiagnostic techniques *viz.*, MAT, ELISA and MSAT were assessed in this study for detecting anti-leptospiral antibodies in patients' sera.

During the study period, a total of seventy five patients were reported to the hospitals in the study area with clinical manifestations suggestive of leptospirosis. All the patients reported with fever and headache. The other common symptoms recorded were myalgia, pyrexia of unknown origin and nausea (PUO). The occurrence of other common symptoms was summarized in Table 1. Primary screening of serum samples was carried out with MAT and the predominant serotype specific antibody in the test sera was detected for *autmnalis* (23/59). Diagnostic relevance and significance of MAT was recognized and reported (Sarvanan *et al.,* 1998) with 71% positivity for specific anti-leptospiral antibody in MAT with the antibody titer in the range of 1:640 to 1:5120. This supports our data (79%) with respect to the detection of anti-leptospiral antibodies with MAT.

Anti-leptospiral IgM titer in ELISA was consistent with MAT test with a marginal increase in seropositivity (83%). Even though, an improvement in titer was seen with ELISA over MAT in terms of higher titer values

Table 1. Percentage of occurrence of clinical symptoms among suspected patients

Sign/Symptom	Percent
Fever	100
Head-ache	100
Myalgia	77
Pyrexia of unknown of origin	36
Nausea	32
Conjunctival suffusion	30
Vomiting	30
Jaundice	23
Splenomegaly	5
Renal failure	-

in few samples, in most cases MAT and ELISA yielded similar titer values. Both the techniques showed high correlation in their anti-leptospiral antibody detection (Figure 5). ELISA and MAT were reported to yield 95% correlation in leptospiral diagnosis (Adler *et al.,* 1981; Natarajaseenivasan and Ratnam, 1996). In fact, ELISA was remarked as a useful alternative test due to its capacity to detect specific anti-leptospiral IgM antibodies (Gussenhoven *et al.,* 1997). Though, ELISA was recommended for routine diagnostic use in serodiagnosis of leptospirosis (Gupta *et al.,* 2004), the low sensitivity of ELISA-IgM (20%) in detecting anti leptospiral antibodies in latent phase sera (Nakarin and Pardutkanchana, 2004) limits its use. Realizing the limitations of MAT and ELISA in leptospiral serodiagnosis, we had examined MSAT as an alternative technique. Data obtained in the present study suggest MSAT as a technique comparable to the gold standard MAT. The technical superiority of MSAT was recognized in detecting anti-leptospiral antibodies by Brandão *et al.* (1998) also.

Analyzing the ELISA and MSAT data, a marginal increase in seropositivity was indeed recorded with ELISA (Figure 5). This may not be considered as a major advantage with ELISA as sporadic false positive results with ELISA were reported (Trepstra and Pereia, 1992). Even though MSAT does not provide quantitative information with respect to antibody titer, its simplicity and inexpensive nature strengthens its diagnostic relevance. When comparing the practical aspects of the three diagnostic tests, the ELISA and MSAT are easier to perform and are also simpler to interpret. The analytical evaluation of the two genus specific tests against the MAT positive sera using heat extracted antigens of

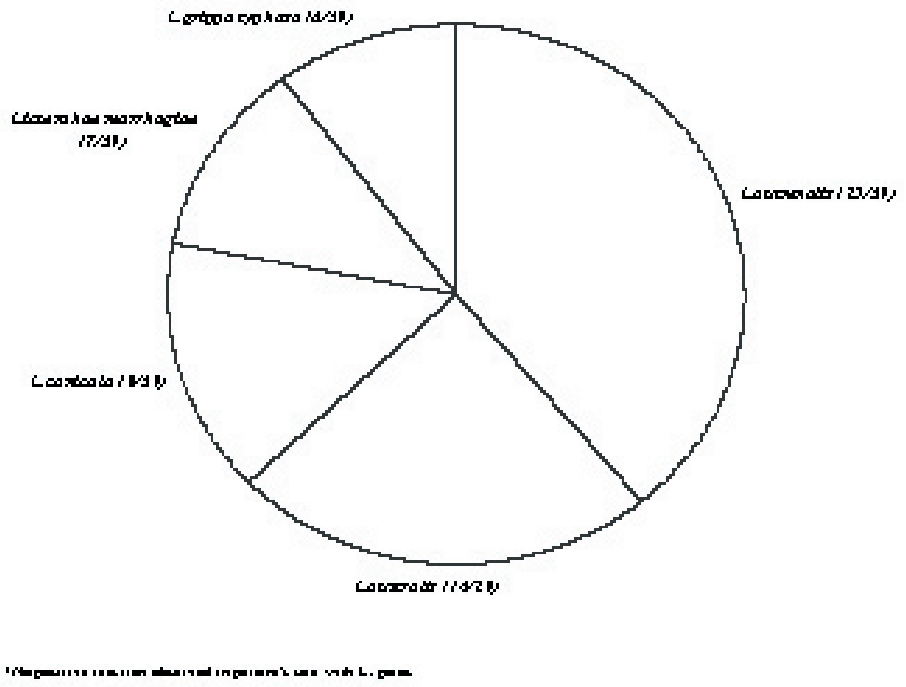

Figure 1. Incidence of different leptospiral serotypes in patients as seen in positive MAT reaction* (n = 59/75)

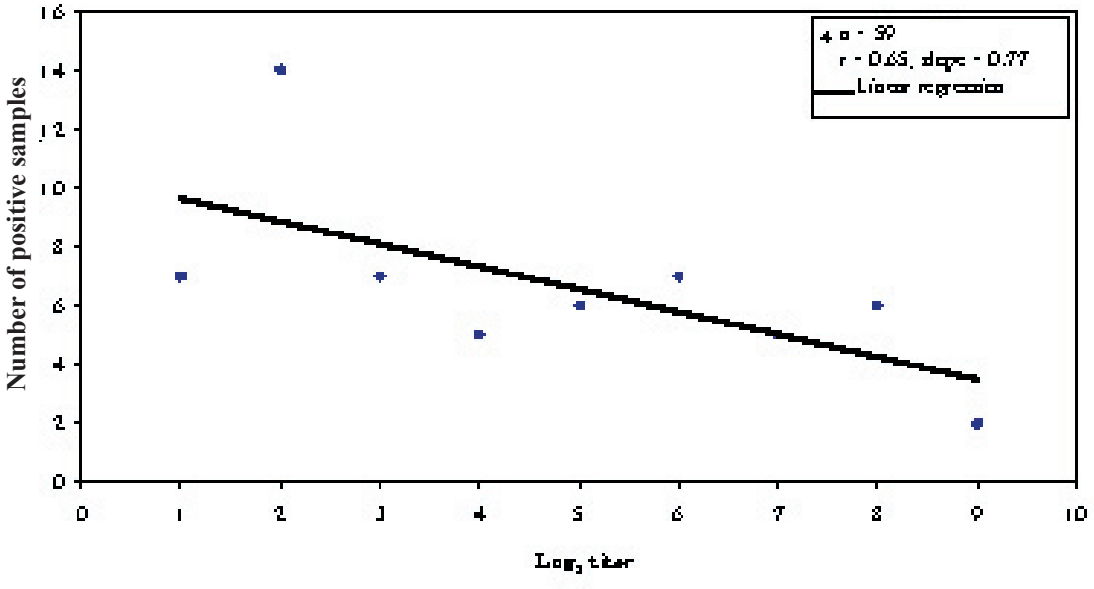

Figure 2. Seropositivity in MAT

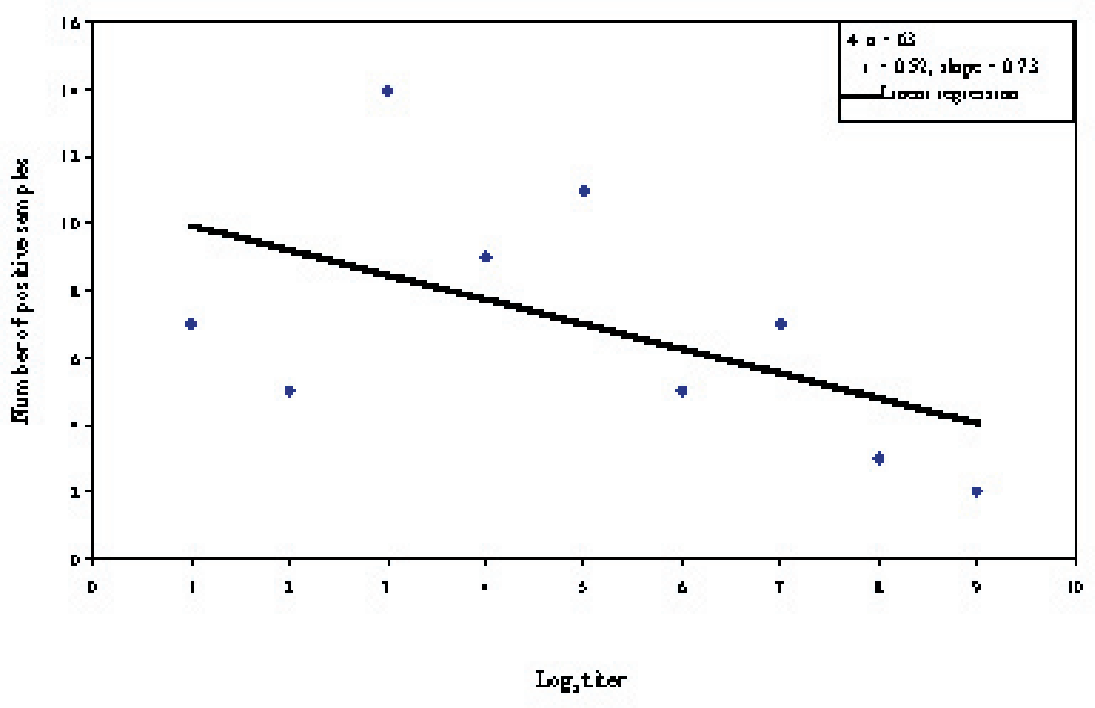

Figure 3. Seropositivity in ELISA

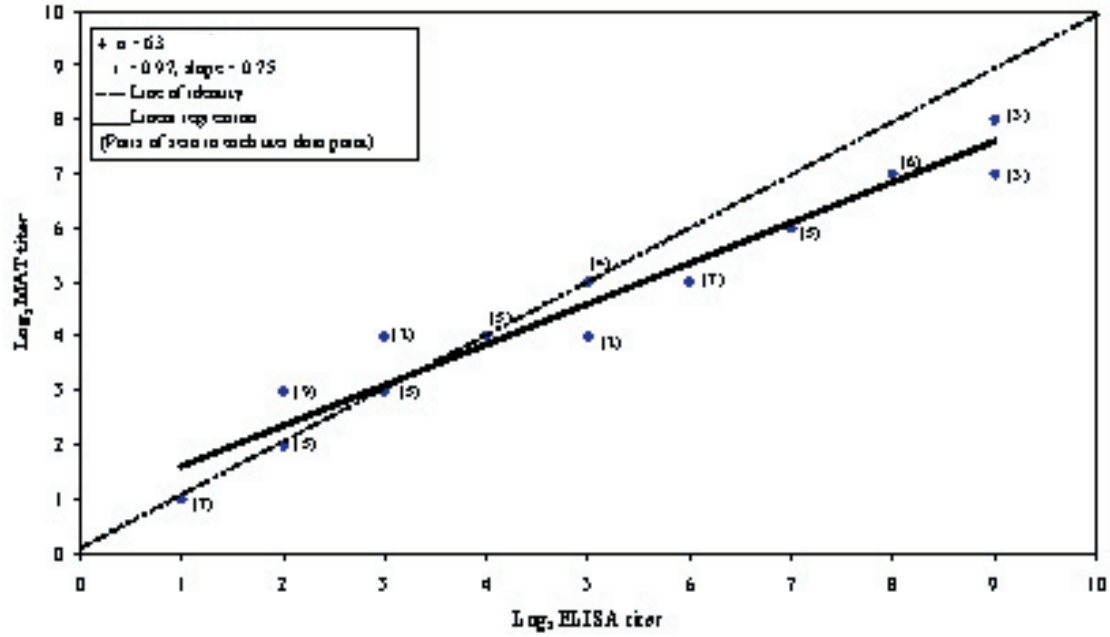

Figure 4. Comparative analysis of anti-leptospiral antibody titer in MAT and ELISA

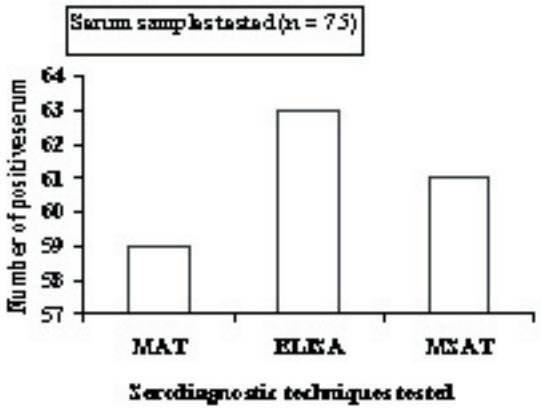

Figure 5. Comparative analysis of three serodiagnostic techniques on seropositivity for anti leptospiral antibodies

australis and *patoc* shows that ELISA had a dependable sensitivity (96.6%) and specificity (62.5%), which was found to be marginally superior to MSAT that exemplifies 94.9 and 68.9% of sensitivity and specificity, respectively. Diagnostic techniques that employ agglutination assay were strongly recommended in the diagnosis of leptospiral infection due to their specificity and inexpensive nature (Chappel *et al.*, 2004). Despite the fact that both ELISA and MSAT exhibited near concordant values in seropositivity, MSAT can be considered as an alternate diagnostic technique in leptospiral diagnosis on the basis of its cost and simplicity.

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