

## Importance of serological analysis - an interpreter of identifying infecting serovar in patients with leptospirosis

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### ABSTRACT

The laboratory diagnosis of leptospirosis is often made using the microscopic agglutination test (MAT), in which live leptospiral cultures in broth is used as antigens representing >10 serogroups undergo reaction with patient serum samples to detect agglutinating antibodies. Data derived from this assay are often used to infer the identity of the infecting leptospiral serovar or serogroup; however paradoxical reactions and cross reactions between the serogroups are quite common. To evaluate the usefulness of this approach, data on culture proven leptospiral cases that occurred in Tamilnadu from January 2010 to December 2013 were reviewed. A total 65 isolates of 5 serovars were identified. The sensitivity of MAT for the prediction of the infecting serovar was determined. Overall the predominant serogroup at a titer of  $\geq 100$  correctly predicted 46% of all serovars isolated. If a titer of  $\geq 800$  was used as the cutoff, sensitivity decreased slightly to 44%. The overall specificity for all serogroups was 68.4%. Serologic analysis appeared to be of little value for the identification of the infecting serovar in individual cases of leptospirosis in humans. Presumptive serogroup reactivity data should be used only to gain a broad idea of the serogroups present at the population level.

**Keywords:** Leptospirosis, serology, MAT

### 1. INTRODUCTION

Leptospirosis is an uncommon zoonotic acute febrile disease caused by pathogenic spirochete of the species *Leptospira interrogans*. The disease occurs throughout the world, but its incidence is highest in tropical regions

including India (Pai and Adhikari, 2001; Prabhu et al., 2009). Initial symptom may be unspecific, oligosymptomatic and having severe spectrum of disease including high temperature, hypotension, multisystemic dysfunction to failure including jaundice and renal failure. Aseptic meningitis was once a common presentation. More recently, pulmonary haemorrhage has been recognized as significant complications in severe outbreaks of leptospirosis. The broad range of clinical manifestations mandates that leptospirosis is part of the differential diagnosis of many febrile illness syndromes (Abboud et al., 2001; Prabhu et al., 2008). In patients with exposure in tropical climates, leptospirosis must be differentiated from malaria and dengue fever, but the differential diagnosis inevitably varies depending on the infectious diseases that are prevalent locally (Prabhu et al., 2014).

The death rate associated with severe cases of leptospirosis may be as high as 20%. Antibiotic therapy with either doxycycline or penicillin has been shown to be effective if initiated earlier (Gulati and Gulati, 2012). It is very difficult to diagnose leptospirosis clinically, highlights the importance of laboratory diagnosis initially dark field microscopy and initiates the antibiotic therapy with low dose of doxycycline and further increased the concentration after receiving culture and serology reports. IgM specific detection assays are now available but these kits may be relied upon to provide a diagnosis after 5-7 days of illness in most cases. The definitive diagnosis is culturing and isolating leptospires that allows for identification of the infecting serovar (Musso and La Scola, 2013; Chaudhry et al., 2013). However, culture is relatively insensitive and requires several weeks' incubation which limits its use in most of the laboratories. The microscopic agglutination test uses a panel of leptospiral strains as antigens for detection of agglutinating antibodies. This assay requires significant expertise to perform and interlaboratory variations in results is also high. Despite these limitations, the MAT has epidemiological value, and it is often used to give an indication of the presumptive serovar of serogroup of leptospires involved in infections (Toyokawa et al., 2011; Prabhu et al., 2009). However, overinterpretation of MAT serologic findings in the absence of a sound knowledge of the locally prevalent serovars may limit its value.

The genus *Leptospira* is classified into several species and classified further into > 200 serovars by using the hybridization technique (Levett, 2003). For convenience, antigenically related serovars are clustered within serogroups. The main purpose of the present study was to evaluate the ability of the MAT to predict the infecting serovar in a population in which leptospirosis has been extensively characterized for four years.

## 2. MATERIAL AND METHODS

Laboratory records was reviewed retrospectively to identify all cases of leptospirosis for which the onset of symptoms occurred during the period of January 2010 to December 2013, as confirmed by isolation of *Leptospira* species obtained from other body sites. Data from these cases were used to determine the sensitivity and specificity of MAT for the prediction of the infecting serovar. The MAT was performed strictly by following the standardized protocol by using a panel of antigens representing both ubiquitous and locally prevalent serovars. The titer value of  $\geq 80$  is considered as positive. The duplicate of MAT procedure was followed if the samples showed the titer value of  $\geq 80$ . The predominant serogroup was defined as a titer of  $\geq 80$  with the maximum titer directed against a single serogroup. Analyses were also performed using a cutoff titer of  $\geq 800$  (Faine, 1982). Cases of leptospirosis were excluded from the analysis if patients were seronegative, if maximum MAT titers of  $< 100$  were detected, if highest titers were detected against *Leptospira biflexa* serovar patoc, if titers of  $\geq 100$  were detected, with equal titers directed against  $> 1$  serogroup.

The sensitivity of the MAT was defined as the proportion of isolates of a single serovar correctly predicted by the corresponding serogroup that was the predominant reactive serogroup in the convalescent phase serum or in the acute phase sample obtained most recently. The specificity of MAT serologic analysis was defined as the proportion of patients with a predominant serogroup whose isolate was of the corresponding serovar. The serovars isolated in the study and their respective species and serogroups are *Leptospira interrogans* (serogroup Australis, serogroup Icterohaemorrhagiae and Canicola).

## 3. RESULTS

During the 4 year period of study, a total of 65 cases of leptospirosis were confirmed by isolation of *Leptospira* species. For the cases, 58 cases were identified to the serovar level. The distribution of serovars among the isolates was as follows: *L. australis*, 31 isolates (53.4%); *L. icterohaemorrhagiae*, 12 isolates (20.7%); *L. canicola*, 8 isolates (13.8%); *L. autumnalis*, 5 isolates (8.7%) and *L. hebdomadis*, 2 (3.4%). Of the 58 patients with isolates identified to the serovar level, 14 died (mortality rate – 24.1%), and for these patients, only acute phase and postmortem serum samples were available. An additional 8 (13.8%) patients had only an acute phase serum sample available. Convalescent phase samples which were obtained a mean of 15 days after the onset of symptoms were available for 34 patients (58.6%). A total of 38 patients had a highest titer of  $\geq 100$  against a single serogroup.

The sensitivity analysis of the MAT serology for the prediction of the infecting serovar was well determined in this investigation. The serogroup Australis was predominant in 31 patients both serology and culturing (100% sensitivity), followed by Icterohaemorrhagiae of 12 patients, Canicola of 8 patients. Overall, the predominant serogroup at a titer of  $\geq 100$ , correctly predicted 27 (46.5%) of all 58 isolates. When the titer of  $\geq 800$  was used as a cutoff, sensitivity slightly decreased to 41.4% (24 of 58 serovar isolates). For patients with serogroup Icterohaemorrhagiae predominating at the titers of  $\geq 100$ , the specificity was 83.3% (10 of 12 serovar isolates). The specificity of the serogroup Canicola was 75% (6 of 8 isolates) and others showed the specificity of zero because no serovars from these serogroups were isolated (Autumnalis and Hebdomadis). Among the patients included in this study, the serogroup of Australis predominating and the specificity also supports the same. The overall specificity was 68.4% (26 of 38 isolates).

The MAT with its still unsurpassed sensitivity and specificity is the gold standard in diagnostic testing of leptospirosis. Unfortunately, the test is difficult to standardize. It requires live *Leptospira* cultures and the estimation of the end point titre is done by eye and thus subjective. For quality assurance it is therefore of utmost importance that the test has an international quality control on its performance (Hartskeerl, 2005). In order to improve the specificity of the prediction of the predominant serovar, the entire procedure should be repeated using convalescent phase samples with the titers of  $\geq 800$ . For that case, only 42 patients possible with the titers of  $\geq 800$ . The specificities determined were Australis 90.4% (19 of 21 isolates), Icterohaemorrhagiae 81.2% (13 of 16 isolates), Canicola 80% (4 of 5 isolates), Autumnalis 0% (0 isolates). The overall specificity was 85.7% (36 of 42 isolates).

#### 4. DISCUSSION

This investigation highlighted the ability of infer the serovar identify of infecting leptospires from the results of serologic testing by use of the MAT was evaluated. For more than one half of the patients, this was not possible. In other studies, serologic data derived from the MAT were often used to infer the infecting leptospiral serovar. This inference may be based on a lack of understanding of the serological relationships among the leptospiral serovars (Oliveira et al., 1997; Kahn, 1982; Lecour et al., 1989; Gollop et al., 1993; Torre et al., 1994; Gerding et al., 1997; Bishara et al., 2002; Levett, 2003; Patarakul et al., 2010; Chou et al., 2012; Chirathaworn et al., 2014).

The agglutination tests for leptospiral antibody were developed immediately after the first isolation of leptospires (some 80 years ago). In the initial period, very few serovars were known and recognized, so it is mandatory to include all the serovars for the leptospiral determination in order to understand the predominant serovars in a particular area. After detection of more serovars it became apparent that serovar specificity was an erroneous concept. Cross reactions between the serogroups are quite common thereby the initial immune response is directed to a heterologous serovar of serogroup. About 50% of the cases included in any study showed paradoxical reactions. The potential for the over-interpretation of serologic data thus is much greater if only acute phase or early convalescent phase serum samples are available for testing.

A broad range of serogroups has been used in the reference laboratories in the MAT to maximize the probability of detecting an immune response to a serovar not expected, either because it has not yet been isolated or because a previously known serovar has been introduced into the population. The co-infections with multiple serovars are also identified as an additional confounding factor. Most of the reports highlighted that the narrow range of serogroups has been used in the MAT test as the panel of antigens which may further reduce the ability of serological analysis to predict the infecting serogroup in a particular area accurately (Cui et al., 1991; Cumberland et al., 1999).

Most of the serogroups contain several serovars. Thus, reaction with an individual serovar selected for use as an antigen representing a serogroup cannot be taken to imply infection with the serovar of the same serogroup. When multiple serovars from a single serogroup are included in the MAT, cross reactivity is the rule rather than the exception. Thus, within a serogroup, antigens of different serovars may not detect identical titers. More studies highlighted the importance of MAT serology and culturing is the standard tool for understanding the epidemiology in a geographical region, as does the identification of the leptospires that cause infection in an individual patient (Saravanan et al., 2014).

Very rarely, a point source outbreak of leptospirosis occurs, allowing for serologic identification of the presumptive infecting serogroup. In some studies, the predominant of Australis identified among 30 of 33 patients and the remaining 3 showed mixed infections. By this it is to be confirmed that extensive testing allowed for the presumptive serogroup to be identified with a high degree of confidence, but it could not identify a probable infecting serovar.

In another study, 2 isolates of different serovars and serogroups identified. The serogroup of one of the isolates was not represented in the MAT panel initially used to test the patient's serum samples and most of the cases showed non reactive to the MAT. When another serovar that was from the same serogroup to which the isolate belonged was included in the MAT antigen panel and the samples were retested, all patients were found to be

seroreactive. In some studies, there will be an interesting observation that the culture showed positive and no antibody response was detected. These data was derived from extensive serologic testing with a panel of MAT antigens that were selected after the identification of isolates obtained from the patients, and they thus represent optimized performance, which is unlikely to be achieved in a laboratory using a restricted range of antigens.

During the past 40 years, leptospirosis has been studied extensively in various places of the world with approximately 30 severe cases of leptospirosis diagnosed every year in each country. Repeated surveillance has identified the appropriate serovars predominantly affecting the individuals and also in the region. The continued existence of a laboratory that performed cultures and identified the isolates allowed for a sufficiently large number of culture proven cases to be evaluated. During this study, new serovars were not identified whereas repeated serovars like Australis, Icterohaemorrhagiae, Canicola, Autumnalis etc.

However, usage of the predominant serogroups in the performance of MAT for the prediction of the infecting serovar is relatively insensitive to various individuals; the prediction matches the serovar from whom the isolates are recovered. This has been low specificity as a predictor of the infecting serovar. Specificity can be increased by applying a more stringent definition of serogroup predominance. Assays which detect IgM are more sensitive than the MAT and give positive results earlier in the acute phase of the disease. 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 the results from paired sera. When only samples from acutely ill patients were considered, the IgM-dipstick assay was of comparable sensitivity to the IgM-ELISA, whereas the sensitivity of the IHA was closer to that of the MAT (Levett et al., 2001).

In addition, an intrinsic limitation of MAT is the subjective interpretation of the results and the difficulties in ensuring standardization between laboratories. So, researchers now are trying to overcome MAT by substituting with some other techniques (Yitshaki et al., 2004). Some studies showed that the MAT is the unreliable tool for the leptospiral epidemiology and usefulness of the Cross adsorption agglutination test (CAAT) and culturing remains the technique of choice to predict appropriately (Smythe et al., 2009).

The MAT derived data cannot discriminate between recent and last *Leptospira* infections, nor can these data be used to determine the severity of the disease. Nonetheless, the data strongly support the presence of human leptospirosis and emphasize the need for a proper diagnosis to ascertain the number of leptospirosis cases among the acute febrile illnesses (Gomard et al., 2014). In general, in house IgM ELISA showed high degrees of sensitivity and specificity than MAT (Natarajaseenivasan et al., 2004).

This zoonotic infection, which is not commonly recognized by medical professionals, as it has protean manifestations and can progress to multi-system failure and death very rapidly. Unlike many other diseases, an effective cure is available and hence it becomes imperative that physicians have a high index of suspicion when confronted with syndromes like fever and jaundice. Along with the sensitization of the clinicians, laboratory support should be strengthened so that the disease can be diagnosed accurately and early. In this investigation, strong laboratory support is important to give direction to the epidemiological investigation. This is usually lacking and neglected in low income countries and needs further strengthening (Sehgal, 2000).

However, the number of samples was less in order to make accurate estimates of indices of accuracy. Leptospirosis has already been included in the proposed integrated disease surveillance program as a disease condition of local interest. Further the extension of this work to all places of the state will provide the accurate data.

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