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ROLE OF MICROSCOPIC AGGLUTINATION TEST (MAT) FOR THE DIAGNOSIS OF LATE /COMPLICATED LEPTOSPIROSIS

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ABSTRACT

Leptospirosis is an endemic spirochaetal zoonosis of worldwide distribution and is underreported due to protean manifestations. It is often mistaken for locally prevalent infectious and non-infectious diseases. It is associated with high morbidity and mortality. Specific diagnostic tools for the disease include blood cultures for leptospires, serologic methods such as microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA), as well as molecular methods, polymerase chain reaction (PCR). Serology remains the mainstay for the diagnosis of leptospirosis as the gold standard MAT is difficult to perform and requires maintenance of live antigens while others are expensive. **Aim:** This study was taken up to know the efficiency of IgM ELISA for the diagnosis of leptospirosis in comparison with MAT at tertiary care centre. **Settings and Design:** The study was carried out from July 2010 to January 2011, during which 151 blood samples were received from suspected cases of leptospirosis. IgM ELISA for leptospirosis was performed with Serion Virion IgM ELISA Kit. MAT was performed using live cultures of leptospira grown on liquid EMJH medium using a battery of 12 antigens as per standard protocol. **Statistical analysis used**: The sensitivity, specificity, positive predictive value and negative predictive value of IgM assay was calculated. **Results**: The sensitivity, specificity, positive predictive value and negative predictive value of IgM assay was calculated using 2x2 tables and found to be 44.44%, 57.37%, 72.13%, 38.88% respectively using MAT as the Gold standard. **Conclusions:** In the present study, MAT was positive in 99 cases (78.65 %) and IgM ELISA in 61 cases (40.4%). The predominant serovar was *L. australis*.

KEYWORDS: MAT, Microscopic Agglutination Test. IgM ELISA, Enzyme Linked Immunosorbent Assay, PCR, Polymerase Chain Reaction, Leptospirosis.

INTRODUCTION

Leptospirosis is a Spirochaetal zoonosis of worldwide distribution.[1] Leptospirosis is greatly under reported due to lack of simple, rapid & efficient tests for early diagnosis. Early diagnosis of leptospirosis is essential because, if untreated, the illness can progress rapidly and, in patients with severe disease, the mortality rates are high. It presents with vague clinical features, thus diagnosis of leptospirosis is always difficult. Its clinical signs and symptoms mimic local prevailing infectious and non-infectious diseases (dengue, viral hepatitis, pneumonia, and cholangitis). Thus, laboratory corroboration of the clinical diagnosis is essential.[2] The conventional tests for its diagnosis include direct microscopy, culture and the most widely used reference standard method, the microscopic agglutination test (MAT). Isolation of leptospires from clinical samples is time consuming and has low success rate of isolation. Dark field microscopy (DFM) is unreliable due to the presence of proteinaceous filaments in the body fluids. Limitations of culture and DFM along with inaccessibility of molecular techniques like PCR, makes serology the mainstay of diagnosis. Microscopic Agglutination Test is the "gold Standard" test but it

requires the demonstration of seroconversion by using paired sera (acute & convalescent serum samples).[3,4] IgM detection for leptospirosis has repeatedly been shown to be more sensitive than MAT when specimen is taken early in the acute phase of the illness towards the end of the first week.[5]

The study was taken up to determine the diagnostic accuracy of IgM ELISA for diagnosing leptospirosis in patients presenting with compatible clinical symptoms, such as fever, muscle pain, increased liver function test (LFT), increased renal function test (RFT) or thrombocytopenia or altogether.

In our study we compared IgM ELISA (Enzyme-linked Immunosorbent Assay) with MAT (Microscopic Agglutination Test) for the diagnosis of leptospirosis in a tertiary care centre, where most of the cases of leptospirosis referred with the complication of leptospirosis.

MATERIALS AND METHOD

The study was carried out from July 2010 to January 2011. During the study period 151 blood samples were received in the Department of Microbiology from patients clinically suspected of leptospirosis. Serum was separated and stored at –20 °C until it was assayed. They were tested for IgM by ELISA for leptospirosis and MAT for confirmation for the diagnosis of leptospirosis.

Procedure for IgM ELISA: All the samples were tested for the presence of specific antileptospiral IgM antibodies by ELISA (Institut Virion Serion GmbH, Warburg, Germany) according to the manufacturer's instructions. All samples with antibody activities more than 20 U/ml were considered as positive.

Procedure for Microscopic Agglutination Test (MAT): MAT was performed by standard procedure[6] by using the following 12 serogroups, *L. australis* (strain Ballico), *L. autumnalis* (strain Bankinang), *L. Canicola* (strain Hond Utrecht IV), L. *Hebdomadis* (strain Hebdomadis), *L. icterohemorrhagiae* (strain RGA), *L. pomona* (strain Pomona), *L. pyrogenes* (strain Salinem), *L. sejroe* (strain Sejroe); *L. grippotyphosa* (strain MoskvaV) *L. tarassovi* (strain Tarassovi) *L. manhao* (L-60) and. *L. biflexa* –semaranga (Patoc I).

All the strains were obtained from National Leptospirosis Reference Centre, Regional Medical Research Centre (WHO collaborating centre for diagnosis in leptospirosis, ICMR) in Port Blair, Andaman and Nicobar islands. These serovars were maintained in liquid EMJH medium (Ellinguahsen McCullough, Johnson and Harris), supplemented with 10% enrichment (Tween 80 and Bovine serum albumin) at 28-30^{0}C in screw capped test tubes. All the serum samples with titers ≥1:80 against at least one pathogenic serovar were considered positive.[6]

RESULTS

Among the 151 patients 61 (40.4%) were IgM positive and 90 (59.60%) were IgM negative. Among these IgM positives, 44(72.13%) were MAT positive and 17(27.86%) negative. The predominant serovar among the MAT positives was *L. australis* (43.18%) followed by *L. grippotyphosa*, *L. pyrogenes*, *L. tarassovi* (11.36%), *L. canicola* (9.09%) and *L. sejroe*, *L. autumnalis* and *L. semaranga* (2.27%).

Among the 90 (59.60%) IgM negatives, 55 (61.11%) were MAT positive and 35 (38.88%) negative. Among these MAT positives, the predominant serovar was *L. australis* (60%) followed by *L. pyrogenes*, *L. tarassovi* (9.09%), *L. canicola* (7.27%), *L. semaranga*, *L. autumnalis* (3.03%) and finally *L. manhoa* & *L. sejroe* (1.81%). Among the 99 (78.65 %) MAT positives the predominant serovar, was one of the serovars associated with "Cane-cutters Disease", *L. australis*, (61.61%), followed by *L. tarassovi* (7.07%), and *L. semaranga* (3.03%). *L. semaranga* tends to react as genus-specific antigen and also agglutinates with human antibodies produced by other infecting serovars, thus detects infections caused by strains which are not yet known to exist in a specific region. It also indicates the existence of previously unknown serovars and thus suggests for the inclusion of more strains in the panel of antigens tested.[7] On the other hand, Weil's syndrome was seen in 38 (25.16%) jaundice was a presenting feature in 5(3.31%) of cases, while in 26(17.215%) complications involving liver, kidneys were seen.

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In the present study, the Sensitivity, Specificity, Positive & Negative Predictive Value of the assay was determined using MAT as reference test and it was 44.44%, 57.37%, 72.13% and 38.88% respectively.

Table. 1: The table is showing distribution of serovars among the MAT positive cases.

Serovar	No of cases	% positivity
L. australis	61	61.61
L. autumnalis	03	3.03
L. canicola	05	5.05
L. grippotyphosa	06	6.06
L. manhao	03	3.03
L. sejroe	02	2.02
L. semaranga	03	3.03
L. pyrogenes	09	9.09
L. tarassovi	07	7.07
Total	99	

Table. 2: The table is showing combined results of IgM ELISA and MAT in serum samples N= 151.

ELISA AND MAT	Total
IgM & Mat Positive	44
IgM & MAT Negative	35
IgM +ve & MAT -ve	17
IgM -ve & MAT -ve	55
	151

Table. 3: The table is showing the relative frequency of presenting symptoms in patients of clinically suspected leptospirosis at the time of admission in the hospital.

Symptoms	No of cases	%positivity
Fever	09	5.96
Fever+ Increased LFT	05	3.31
Fever+ Increased RFT	32	21.19
Fever+ Increased LFT Thrombocytopenia	16	10.59
Fever+ increased LFT+RFT	26	17.21
Fever+Increasd LFT+Thrombocytopenia	18	11.92
Fever +Thrombocytopenia	07	4.63
Fever+weil's syndrome(Increased LFT+Increased RFT+thrombocytopenia)	**38**	25.16
Total	151	

Table. 4: The table is showing the no of cases with other infections at the time of admission in the hospital (N=151).

	No of cases	IgM		IgM		%positivity
		P	N	P	N	
HbsAg+ve	3	1	2	1	2	1.98
Dengue +ve	3	1	2	3	0	1.98
Dengue & HbsAg+ve	2	2	0	2	0	1.32
HCV+ve	1	nd	nd	1	0	0.66

nd- not done

DISCUSSION

Leptospirosis, the most widespread zoonosis, is emerging as a major public health problem. Leptospirosis occurs as two clinically recognizable phases. The most common and early phase is anicteric leptospirosis, a self-limited illness which occurs in 85% to 90% of the cases. The second is the immune stage or the Icteric leptospirosis. Weil's syndrome is a more serious, potentially fatal, syndrome which occurs in 5% to 10% of the cases.[7]

The clinical manifestations of human leptospirosis are diverse, ranging from mild, flu-like illness to a severe disease form known as Weil's syndrome. Severe disease is characterized by jaundice, acute renal and hepatic failure, pulmonary distress and hemorrhage, which can lead to death.[8]

If the illness is not treated within the first 2-3 days, it may progress to severity. Meningitis, anuira, irritis, liver failure and toxic delirium may develop depending on the infecting serovar and other factors. The severe form of illness may be fatal death being due to renal failure. Usually the patient recovers after a prolonged convalescence.[7]

Thus early diagnosis of leptospirosis is essential because, if untreated, the illness can progress rapidly and, in patients with severe disease, the mortality rates can be high. Some untreated patients can develop kidney damage, meningitis, liver failure, and respiratory distress and in rare cases death occur.[9] The diagnosis based on clinical symptoms is not reliable; therefore, laboratory support is an important tool in the diagnosis of the disease. Because the culture of the organism is time-consuming and expensive, several rapid Assays have

been developed recently that can be used for screening of acutely ill patients.[9]

Direct diagnosis using dark field microscopy is very difficult and does not have sensitivity and specificity. Therefore, serological diagnosis is the best alternative. Detection of IgM against surface antigens is possible after day 5 of the disease onset. MAT is an available and the most reliable reference assay in diagnosis of acute leptospirosis.

Among several other serological methods that were introduced for the early diagnosis of acute leptospirosis including the slide agglutination assay, IHA (Immuno heamaggglutination Assay) immunofluorescence, and ELISA, the latter is easier, more reliable, and most commonly used. But the sensitivity of ELISA is mostly related to the time of blood sampling.[5]

The ELISA assays has been employed in many studies. In a study conducted on 108 serum samples from patients with leptospirosis and 245 seronegative samples as a control by Brandão and colleagues, Sensitivity and Specificity of this IgM ELISA assay was reported to be 90 percent[10] and in a study conducted by Smiths and colleagues showed, 85.5% sensitivity & 97.9% specificity.[11]

Several studies conducted by Cinco M *et. al*. (1992), Gussenhoven G. *et al*. (1997), Levett P N and Whittington C U (1998), Terpstra WJ *et al.* (1980), Winslow W E, *et al*, (1997) and Yersin C *et al* (1999) reported the Sensitivities ranging from 68 to 100% for various ELISAs.[12-17]

Vital and co-workers have also used the IgM ELISA assay on 19 MAT confirmed samples isolated from patients with leptospirosis and reported a 100% Sensitivity and Specificity.[18]

In a comparative study, Ooteman and colleagues investigated 125 samples from patients with leptospirosis using MAT, polymerase chain reaction, and IgM ELISA techniques. The IgM ELISA assay showed 96.6% sensitivity and 93.3% specificity.[19]

In the present study, the second - generation assay, a commercial quantitative IgM ELISA assay (Serion-virion) was used for diagnosis of acute leptospirosis. The sensitivity, specificity, positive and negative predictive value of the assay was determined using MAT as reference test and it was 44.44%, 57.37%, 72.13%and 38.88% respectively.

Our results are comparable with the study conducted by Bajani MD (2003), where in for acute phase sera the sensitivity was low between (38.5 to 52.7%), and for convalescent phase sera was much higher (67.2 to 93.8%) was reported.

The sensitivity and specificity of ELISA are dependent on many parameters, particularly the time of sampling and the coating antigen. Since leptospirosis is an acute bacterial disease, the diagnosis is based on detection of antigen-specific IgM that is detectable 6 days after the onset of infection. Thus, the sensitivity of IgM ELISA is low during the first week of infection but increases thereafter.[5]

Sensitivity and specificity of the assay could be improved by the coating of pure and specific antigens isolated from one or preferably multiple locally dominant pathogenic species, instead of the standard or non-pathogenic bacterial isolates.[5]

The main reason for attempting an early diagnosis of leptospirosis is to facilitate appropriate treatment, particularly for selection of an appropriate antibiotic treatment. The diagnosis can be guided by laboratory test results because some common infectious diseases are listed in the differential diagnosis of leptospirosis. A limitation to the use of single serum samples for sero-diagnosis (such as our study) is the persistence of the antibodies. Antileptospiral IgM antibodies are also persistent, but the rate of the decline shows marked variation.[20] Thus, a single IgM positive sample taken during an acute febrile illness with symptoms suggestive of leptospirosis is presumptive evidence of infection, but this finding requires confirmation by testing a convalescent sample, preferably by the use of an alternative method.[20]

Sensitivity of the serodiagnostic assays in acute-phase disease is very important. IgM antibodies have been detected as early as the second day after the onset of symptoms, while IgG antibodies are detectable in the 7th day of the illness.[1]

Furthermore, as MAT detects both IgM and IgG and lacks sensitivity and specificity when early acute-phase serum specimens alone are tested rather than paired specimens.[1] Patients with fulminant illness may die before seroconversion occurs. A MAT may also be less sensitive than an IgM ELISA, even for convalescent-phase specimens.[8]

The lower sensitivity of IgM ELISA in our study could be due to referral bias as ours being a tertiary care hospital, most of the patients in this study were referred for the management of complications. Hence these cases were not in acute phase of leptospirosis where IgM ELISA is more sensitive.

Among the 151 cases, 3(1.98%) cases were HBsAg+ve, 2(1.32%) cases were Dengue positive, and 2 cases (1.32%) showed co- infection of dengue & HBs Ag, while 1 (0.66%) case was HCV positive. This is in accordance to the data as shown in table 3, thus the deranged LFT/RFT /thrombocytopenia/Wiel's syndrome could be chiefly the complications of leptospirosis.

Therefore those cases were not in acute phase of leptospirosis during which period IgM ELISA is more sensitive.

CONCLUSION

From this study it can be concluded that though IgM ELISA is a sensitive test for the diagnosis of leptospirosis but it fails to detect cases which present late in the course of the disease with various complications and in these circumstances MAT can be more informative.

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