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Research Article

COMPARISON STUDY BETWEEN IN-HOUSE IGM DOT-ELISA AND THE MICROSCOPIC AGGLUTINATION TEST (MAT) FOR THE DIAGNOSIS OF HUMAN LEPTOSPIROSIS

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ABSTRACT
An indirect enzyme-linked immunosorbent assay (ELISA) was compared with the microscopic agglutination test (MAT) for the diagnosis of bovine leptospirosis. Blood samples from a total number of 319 HBsAg negative suspected leptospirosis case's were received from Government Hospital and from a few private hospitals of Salem district, Tamilnadu, India. The serum samples were examined for the presence of anti leptospiral antibodies using a commercial qualitative method of an in-house Dot-ELISA assay and the results were compared with WHO standard Microscopic Agglutination Test (MAT). The following interesting results were noted, 132 (41.7 %) serum samples were positive to Dot-ELISA, while 130 (40.7 %) were positive to MAT. All samples positive to MAT were positive to Dot-ELISA, on of the samples were positive for MAT and negative to Dot-ELISA. The Dot-ELISA showed 100% sensitivity compared to MAT. The current diagnostic Dot-ELISA appears as a rapid, non hazardous and better alternative to MAT for the diagnosis of human *Leptospirosis*.
KEYWORDS: Zoonosis, *Leptospirosis,* In house Dot-ELISA, MAT

INTRODUCTION

Leptospirosis is a major public health problem worldwide Zoonoses, particularly in the tropics[1]. Leptospirosis is considered an emerging infectious disease in most part of Asia especially in countries like Thailand[2] and India. The clinical presentation of *leptospirosis* in humans is variable, and can range from a mild flu-like illness to a severe disease with pulmonary hemorrhage, renal failure, and occasionally death[3]. Consequently, *leptospirosis* is easily mistaken for other febrile illnesses including influenza, dengue fever, meningitis, or hepatitis. Therefore, rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations for optimal treatment and patient management. Laboratory confirmation of human l*eptospirosis* relies mainly on serological assays aimed at the detection of specific antibodies in serum samples. The microscopic agglutination test (MAT) is considered the standard serologic test that is specific and provides useful epidemiologic data in the form of presumptive serogroups [4]. However, this assay is not suitable for routine laboratories since it is technically demanding, costly, and requires the maintenance of live, hazardous stock serovar cultures and also requires analyses of paired sera to verify the seroconversion which delays the diagnosis [5]. Ideally, a diagnostic test should be easy to perform, rapid and use only a single specimen [6]. Some potentially useful screening tests for use in all routine laboratories have been proposed. Among these serologic approaches, enzyme-linked immunosorbent assay (Dot-ELISA) for both IgG- and IgM-leptospiral antibodies have been developed [7] and several commercial test kits are available [8], mostly using broadly reactive *Leptospira* antigen obtained from nonpathogenic *L. biflexa* serovar Patoc-I. However, the use of this serovar may affect the sensitivity of testing in some regions where different leptospiral serovars predominate that do not induce antibodies that cross-react with serovar Patoc-I [9]. Most of the tests aimed for the detection of leptospiral IgM is detectable from about the 2nd- 5th day of symptoms [10] that can help in the rapid diagnosis of the disease by using a single serum sample. In the present study, a test strip was prepared using antigen from prevalent serovar in a form suitable for diagnostic format. The test strip was evaluated and compared with the standard MAT using single serum samples from patients with known MAT titers and also with a commercially available IgM ELISA.

MATERIALS AND METHODS

Subject

319 suspected leptospirosis case's age ranging from 1 to 70 (Table: 1) were received from Government Hospital and from a few Private Hospitals of Salem district. All the samples were found to be HBsAg negative by the respective hospitals.

Microscopic Agglutination Test (MAT)

The antigens used with MAT were made up of 4-8 days-old live cultures of the following serovars: *L. australis, L. autumnalis L. icterohemorrhagiae, L. canicola,* and *patoc* strain *patoc-1*. All the strains received from FAO/WHO collaborating centre for diagnosis in leptospirosis Brisbane, Australia. MAT was performed as described by [11] with some modifications.

Each serum sample was initially diluted (1/25) with phosphate buffer saline (PBS) pH 7.2 in a microtitre plate (Greiner Labrotechnik), 25 µl of PBS were added into each well of the plate and an equal volume of the diluted serum sample was placed in the first row (A row) of the plate. The diluted serum was serially diluted (two-fold). Then, 25 µl of the live antigens (4-8 days-old cultures containing 10^8 leptospires/ml) were added to each well. Thus, each well contained an equal volume of the diluted serum sample and the antigen. For each serum sample tested, there were eight dilutions ranging from 1/100 to 1/12,800. The plate was gently shaken for 15-20 seconds to mix the contents, then

covered to exclude debris and prevent evaporation, and then incubated for one hour at 37°C. The test was read by transferring a drop from each well onto a glass microscope slide. The drops were examined by dark-field microscopy (x200). A positive reaction was regarded as one in which 50% or more of the antigens (live leptospires) were agglutinated. The titer end point was taken as the last well in which 50% or more agglutination was observed.

In-House IgM Dot- ELISA

Preparation of the test strip

Sonicated antigen was prepared from *Leptospira interrogans* serovar *L.autumnalis* as per [12] with a few modifications. Briefly, the organism was cultivated in (or EMJH) medium, and incubated at 30°C with shaking for 7 days to yield a cell density of about 108 cells/ml. The organisms were killed with 0.5 mg/L sodium azide, and disrupted by sonication at 20 kHz for 3 periods each of 3 min.

The sonicated leptospiral antigen was diluted in 0.05 M carbonate buffer (pH 9.6) and 2 µl (protein concentration, 0.3 µg/2µl) was dotted onto a strip (0.8 cm X 2.5 cm) of nitrocellulose (NC) membrane. After being air dried, the paper strips were treated with blocking buffer (PBS, pH 7.2 containing 0.1% Tween 20[PBS-T] and 5% bovine serum albumin [BSA] for 30 min at room temperature. The paper strip was air-dried and was then stored in a small sealed plastic bag at 4°C until used for testing.

The test strip was numbered with the corresponding serum numbers. The test was performed in a 2 ml-microtube by adding 20 µl of serum to 200 µl of PBS-T buffer (PBS, pH 7.2, containing 0.1% Tween 20) to make a dilution of 1:10. Once the serum was mixed by gentle shaking the micro tube, a test strip was added. The micro tube was placed down horizontally, and left at room temperature for 30 min with gentle shaking. Each test strip was taken out and washed with PBS-T in the same container for 15 min, twice. The strips were then incubated with horseradish peroxidase (HRP)-conjugated antihuman IgM (AB gene UK) (1:1000 in PBS-T containing 1% BSA) for 30 min, then washed twice with PBS-T for 10 min, and developed with Tetra methyl Benzidine (TMB) (Genei, Bangalore, India) for 15 min in dark. The reaction was stopped by rinsing the strips with PBS. Appearance of blue colour dot on the nitrocellulose membrane indicated positive result. Non appearance of blue colour dot on the nitrocellulose membrane indicated negative result.

RESULTS

Out of the 319 serum samples examined by MAT, 130 (40.7 %) were positive to MAT, while 189 (59 %) were negative (Table 1). Results based on current In-House Dot-ELISA, 132 (41.3%) were positive, while 187 (58.6 %) were negative (Table 2). The sensitivity of the test strip IgM dot-ELISA assay was 100%, its specificity was 98.9%, its PPV was 100 % and its NPV was 98.9%. By comparing MAT and ELISA, 132 (41.3%) were positive to ELISA, while 130 (40.7%) were positive to MAT. The IgM dot-ELISA assay showed comparable results, the rapidity, simplicity, single sample testing without any technical expertise makes this test more suitable as a rapid screening test.

DISCUSSION

Leptospirosis has been under diagnosed and under reported in India due to the lack of awareness of the disease, inadequate epidemiological data and unavailability of appropriate laboratory diagnostic facilities in most parts of the country [13]. Serodiagnosis of leptospirosis by an IgM-specific ELISA assay is often used as an alternative to MAT in routine diagnostic laboratories [6]. The MAT detects both IgG and IgM antibodies [14] but the MAT titers are usually low during the acute stage of the disease and, hence, diagnosis based on a single serum sample is difficult [5].

Detection of IgM antibodies by ELISA is more sensitive than the MAT and gives a positive result earlier in the acute phase of the disease. It is easier to perform, can easily accommodate a large number of samples and, gives a less subjective result than MAT[10]. The performance of conventional ELISA is hampered due to the limited shelf-life of reagents and the requirement of technical expertise.

To overcome these problems, simpler versions of ELISA such as dot-ELISA have been developed in many laboratories[15] due to the use of smaller volumes of reagents, the possibility of visual readings and no requirement of special equipment. So this method can be used to diagnose leptospirosis in peripheral laboratories with relatively little expertise. Comparative evaluation of several commercial test kits for use as rapid screening methods for serodiagnosis of acute leptospirosis in different countries [16] showed the variability in screening test sensitivities and specificities.

The screening test's sensitivity in any given setting is dependent on the ability of test antigens to detect antibodies produced against the site-specific leptospiral serovars. Hence, laboratories need to validate the performance of these screening tests for use in the setting [17]. So, in the present study, an IgM-Dot ELISA in a strip form for the detection of leptospire specific IgM antibodies has been standardized and evaluated. In this test, antigen was used from the locally prevalent leptospiral serogroup, in a form suitable for diagnostic format which could be done on single or large number of samples for rapid detection of specific IgM antibodies in human leptospirosis.

The In-House IgM dot-ELISA and WHO standard MAT test positive result observed and were given in table 2 and 3. Two serum samples negative in MAT but positive in the IgM-dot ELISA assay (sensitivity, 100%). In the present study the assay was found to be sensitive for infections with strains of several serogroups by using these MAT-positive serum samples (serum titers ≥1:100). These serogroups included *L.australis, L.autumnalis, L. icterohemorrhagiae, L.canicola,* and *patoc* strain *patoc-1*. However, knowledge of the serogroup has no clinical implications.

Specificity was found to be 98.9 % in the test strip IgM-dot ELISA compared to the MAT test. Two false positive reactions i.e. Dot ELISA-positive/MAT-negative were obtained from 2 patients. The IgM antibody detected in these patients could be persisting antibody due to previous leptospiral infection or cross-reacting antibody. In this study, the test strip IgM dot-ELISA assay performed with sera at 1:10 dilution, since this dilution of patient serum was found to give best results. The antigen used for this test was a crude sonicated preparation of endemic pathogenic leptospiral serovar.

The Dot-ELISA evaluated in this study offered good negative predictive values 96.00%, thus making the test ideally suited for rapid screening. The positive predictive value (PPV) of the dot ELISA was 97.33 %. The use of a single serovar to prepare the antigen for dot ELISA appeared reliable and easy. This antigen was stable and could be stored for a long time either in liquid state or coated onto NCP. On the other hand, MAT used live leptospires belonging to different serovars, which had to be propagated and subcultured continuously to carry out the test. This was very tedious and created a risk of

infection to laboratory personnel. Dot ELISA showed 100% sensitivity compared to MAT.

CONCLUSION

In conclusion, the test strip IgM dot-ELISA assay using locally prevalent leptospiral antigen offered good sensitivity and specificity; yielding accurate results comparable to the reference MAT. The assay was simple, inexpensive, and easy to perform, with visual reading of the results that do not require special equipment. Performing the assay was also easier, either by a single assay format or even for assessing larger number of specimens, more importantly within two hours time. Thus, it could be used as an initial screening test for leptospiral infection, with subsequent confirmation of positive test results by MAT.

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Table 1: Age and sex wise distribution of Leptospirosis among suspected cases.

S.No	Age groups	Male	Female	Total
1	01 to 5	56	47	103
2	06 to 10	49	51	100
3	11 to 15	37	22	59
4	16 to 20	04	02	06
5	21 to 25	07	05	12
6	26 to 30	10	05	15
7	31 to 35	01	03	04
8	36 to 40	02	02	04
9	41 to 45	03	01	04
10	46 to 70	10	02	12
	Total	179	140	319

Table 2: Leptospiral seropostivity among suspected cases from Salem District by MAT.

S.No.	Age groups	Total samples	Male		Female		Total Positivity
			Tested	Positive	Tested	Positive	
1	01-05	103	56	19	47	25	44
2	06-10	100	49	22	51	21	43
3	11-15	59	37	17	22	07	24
4	16-20	06	04	Nil	02	01	01
5	21-25	12	07	02	05	03	05
6	26-30	15	10	03	05	01	04
7	31-35	04	01	01	03	01	02
8	36-40	04	02	01	02	02	03
9	41-45	04	03	01	01	Nil	01
10	Above 46	12	10	02	02	01	03
Total		319	179	68	140	62	130 (40.7%)

Table 3: Leptospiral seropostivity among suspected cases from Salem District by In-house dot-ELISA.

S.No.	Age groups	Total samples	Male		Female		Total Positivity
			Tested	Positive	Tested	Positive	
1	01-05	103	56	19	47	25	44
2	06-10	100	49	22	51	21	43
3	11-15	59	37	17	22	07	24
4	16-20	06	04	Nil	02	01	01
5	21-25	12	07	02	05	03	05
6	26-30	15	10	03	05	01	04
7	31-35	04	01	01	03	01	02
8	36-40	04	02	03	02	02	05
9	41-45	04	03	01	01	Nil	01
10	Above 46	12	10	02	02	01	03
Total	**319**	**179**	**70**	**140**	**62**	**132 (41.3%)**	

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