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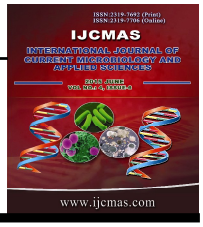

Original Research Article

Dark field Microscopy an important conventional technique for the early diagnosis of Leptospirosis

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Keywords

Dark field Microscope (DFM), ELISA, Leptospira

A B S T R A C T

Leptospirosis is a zoonotic disease caused by pathogenic species of genus *Leptospira*. Timely diagnosis is important as clinical symptoms are often nonspecific which is similar to several other febrile conditions. We aim to study criteria that can be used for early diagnosis by performing Dark field microscopy and PANBIO IgM ELISA. A total of 148 blood samples were collected from the suspected patient.DFM was performed to demonstrate *Leptospira* in the blood after differential centrifugation. PANBIO ELISA was done for *Leptospira* IgM antibody in the serum samples. By using single blood samples from the patient, we have found that sensitivity of DFM was 34.45 % compare to PANBIO IgM ELISA which was 29.05%. Sensitivity of DFM was further increase to 49.05% when the samples were collected fresh from the patient having infection duration less than 7 days. This study shows the validation of DFM results by PANBIO ELISA for *Leptospira* IgM antibody, based on which we recommend that dark field microscopy can be very useful for early and rapid diagnosis of leptospirosis

Introduction

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. The spectrum of human disease caused by leptospires is extremely wide, ranging from subclinical infection to a severe syndrome of multi organ infection with high mortality [1].Icteric leptospirosis with renal failure, was first reported over 100 years ago by Adolf Weil in Heidelberg, Germany[2]. Leptospirosis usually results from contact with urine of infected animals [3]. Diagnosis is mainly based on serological tests, with the microscopic agglutination test (MAT) considered as gold standard [4]. Prompt identification of leptospirosis is needed, as antibiotic therapy provides the greatest benefit when administered early in the infection stage [5]. Timely diagnosis relies on an effective laboratory test, since the presentation of early disease is often nonspecific [6]. Misdiagnosis is a major problem in regions where other causes of undifferentiated febrile illness and hemorrhagic fever are endemic [7].The microscopic agglutination test (MAT), the

standard for confirmation of diagnosis, is impractical for clinical decision making since it requires analysis of paired sera for proper interpretation and a reference laboratory to perform dark-field microscopy [8].Wolf noted that dark field microscopy (DFM),after differential centrifugation of Ruy, may enhances the chance of seeing Leptospira and thereby making diagnosis possible[9]. Chandrasekaran *et al* used 1% solution of Na-oxalate in sterile saline and phosphate buffered 1% sodium oxalate solution (pH 8.0) to demonstrate *Leptospira* spp. in varying concentrations in the blood of patients and police dogs [10]. Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy [11]. In this study we have evaluated early diagnosis of leptospirosis by performing dark field microscopy and PANBIO IgM ELISA.

Materials and Methods

This study was done at two different places. One part of the study was done at Annapoorna Medical College and Hospital Salem Tamilnadu where 67 patients between the age of 13 and 65 years from medical wards (both outpatient and inpatients) of Annapoorna Medical College and Hospital, Vinayaka Mission Hospital and Vinayaka Mission superspeciality Hospital, Salem were included for the study during August 2011- July 2013. Second part of the study was done at Patna Bihar where 81 patients were included for the study during May 2014-January 2015.Patients were included based on undifferentiated febrile illness of ≤ 3 weeks duration with other symptoms based on criteria designed by Faine et.al [13]. Clinical and demographic data were collected from the patients using a questionnaire. After taking verbal informed consent, blood samples (5ml each) was collected aseptically in to two sterile SV10 vials, one containing 500 µl 1% sodium oxalate solution pH 8.0 and another plain tube. The former sample was used for dark field microscopy. The other was used for serodiagnosis by PANBIO IgM ELISA.

Preparation of phosphate buffered 1% sodium oxalate solution

Phosphate buffer pH 8.0 was prepared as per the method given by Wilkinson [12]. To 100mL of these 2 grams of sodium oxalate crystals is added. The mixture was then made up to 200mL with distilled water. The solution was autoclaved and distributed in SV1O vials (Laxbro) each vial containing 500 µl aliquot. The vials were kept in the refrigerator at 4°C.

Dark field microscopic examination (DFM)

The freshly collected blood in sodium oxalate solution was centrifuged at about 3000 rpm for 5 minute to sediment the cellular elements. The supernatant plasma (10 µl) was placed on a 1mm thick new microscopic slide. A cover slip was placed on the drop and pressed to form a thin film without air bubbles. Using the high power objective (x400) of a dark field microscope (Olympus CX-41) one edge of the film was focused to see the *Leptospira*. The number of *Leptospira* seen is determined by simple counting and the report was given as *Leptospira* positive per HPF or per 100 HPFs depending upon the concentration. If no *Leptospira* was seen in 100 HPFs after high speed centrifugation, the report was given as *Leptospira* negative.

Panbio IgM ELISA

Standard ELISA procedure was followed as per the manufacturer instructions. Optical density (OD) values were recorded in an ELISA reader by using 405 nm filters. The

mean OD value of standard serum was substracted from the OD value of substrate blank. The OD value of test sera after substraction from the OD value of substrate blank were referred to the selected table to find out the corresponding value of IgM *Leptospira* antibody content in IU/ml. More than 15-20 IU/ml was considered to be positive for IgM *Leptospira* antibody in the test sera.

Result and Discussion

Table 1 shows clinical categorization of suspected leptospirosis cases. It has been found that out of 148 cases of different age ranging from 13- 65 years 96 (64.86%) were male and 52(35.13%) were female. The most common symptoms found was fever and headache followed with myalgia and jaundice.

Table 2 shows relation between the result of Dark field microscopy (DFM) and ELISA. It was found that there is a greater chance of detecting *Leptospira* 34.45 % (51/148) by DFM compare to 29.05% (43/148) by IgM antibody ELISA. Their combined efficacy was found to be 63.51% (94/148).

Table 3 depicts decrease of sensitivity of DFM from 49.05% (26/53) in early infection of 1-7 days to 21.21% (7/33) in infection of >15 days. However sensitivity of PANBIO ELISA was found to be 33.87% (19/62) in compare to DFM sensitivity of 29.03% (15/62) when infection was of 8-14 days

Table.1 Clinical categorization of suspected cases

Clinical category	Sign and symptoms	Number of cases from Salem		Number of cases from Patna	
		Male	Female	Male	Female
I	Pyrexia, headache, myalgia, vomiting, lethargy, hepatomegaly	26	7	41	27
II	Pyrexia ,headache jaundice with abnormal LFT, hepatomegaly	15	9	5	4
III	Pyrexia ,headache altered sensorium, conjunctival suffusion ,meningitis	2	1	2	1
IV	Pyrexia, headache, Abnormal renal function test with glomerulonephritis	4	3	1	0
	Total	47	20	49	32

Table.2 Comparison of DFM and PANBIO IgM ELISA by using single blood sample

Clinical category	Total number of cases	DFM + IgM ELISA -	DFM + IgM ELISA +	DFM - IgM ELISA +	DFM - IgM ELISA -
I	101	29	3	27	42
II	33	14	0	10	9
III	6	2	0	1	3
IV	8	3	0	2	3
Total	**148**	**48**	**3**	**40**	**57**

+ Positive, - Negative

Table.3 Sensitivity of DFM and PANBIO IgM ELISA on single blood sample

Duration of infection (days)	Total number of cases	DFM + IgM ELISA -	DFM + IgM ELISA +	DFM - IgM ELISA +	DFM - IgM ELISA -	DFM Sensitivity (%)	ELISA Sensitivity (%)
1-7	53	26	0	10	17	49.05	18.86
8-14	62	15	3	19	25	29.03	33.87
≥15	33	7	0	11	15	21.21	33.33

+ Positive, - Negative

In this study we have found that sensitivity of DFM is 34.45% and it was as high as 49.05% when the sample was collected fresh from the patient having infection duration of 1-7 days Kanchan Sharma during the study on the early diagnosis of leptospirois has found that the sensitivity of DFM (60.5%) and combined efficacy of ELISA and DFM was 96% [14].we have used 1% sodium oxalate solution as anticoagulant to collect blood sample. Chanderasekaran has revealed that there is great chance of detecting *leptospira* by dark field microscopy when the sample was collected fresh and he found 64.7% correlation between DFM and *Leptospira* IgM antibody ELISA [15].

Sensitivity of DFM decreases to 29.03% when it was collected during 8-14 days of infection which further decreases to 21.21% of infection duration of more than 15 days. We found 1-5 *Leptosira* per HPF under dark field microscope. It is very important to give adequate time to locate live *Leptospira* with characteristic morphology and motility under dark field examination however probability of misinterpretation of cellular elements with similar morphology in the sample can not be ignored. We do believe that result of DFM needs to be correlate with clinical findings and molecular test like PCR.

Although microscopic agglutination test agglutination test may be consider as gold standard method, 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[16]. Considering the value of cost effectiveness, simple reliable and rapid to perform we suggest dark field microscope can be used as an important tool for the early diagnosis of leptospirosis.

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