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TO FIND OUT THE PREVALENCE OF LEPTOSPIRAL INFECTION IN PATIENTS OF PYREXIA OF UNKNOWN ORIGIN, JAUNDICE, BY IMMUNOLOGICAL AND MOLECULAR TECHNIQUES AND THEIR COMPARISON – A PROSPECTIVE STUDY.

Pathology

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ABSTRACT

INTRODUCTION :- Leptospirosis is caused by infection with pathogenic spirochaetes of the genus Leptospira. It could be diagnosed with the help of Dark ground microscopy, in- vitro culture, serology and molecular method, each one with its merits and de- merits.

AIMS & OBJECTIVES :- To find out the prevalence of leptospiral infection in patients of pyrexia of unknown origin, jaundice, by immunological and molecular techniques

MATERIALS & METHOD:- In this study 110 urban – rural patients with history of pyrexia of unknown origin or jaundice were selected.

CONCLUSION:- In serological tests IgM positivity was found in 7 cases in pyrexia with jaundice group. Leptospira serovar autumnalis was found to be the most common that is 13 case, followed by serovar canicola 3 cases and icterohaemorrhagiae in 1 case.

KEYWORDS

INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic spirochaetes of the genus *Leptospira*.T h e disease is maintained in nature by chronic renal infection of carrier mammals, which excrete the organism in their urine (Faine et al, 1999). Humans become infected through direct exposure to infected animals or their urine, or through indirect contact via contaminated water or soil contaminated with urine of a Leptospira shedder (Levett, 2001).

Rodents are an important reservoir of Leptospira but mice, sheep, cattle, pigs, dogs, racoons, goats, marsupials, and bats have been reported as possible reservoirs (Faine et al, 1999, Levett, 2001). The usual portal of entry is abraded skin or intact mucous membranes. The spirochetes then travel to the liver where they reproduce (Martinez et al, 2000, Luks et al, 2003).

Human infection by pathogenic *Leptospira* may present variable clinical manifestations ranging from subclinical infection with undifferentiated febrile illness to jaundice, renal failure, and potentially lethal pulmonary disease (Bharti et al, 2003). Leptospirosis is typically a biphasic illness (anicteric), but a fulminant disease (icterohaemorrhagic form, Weil´s Disease) can be found in 5–10% of all patients. Fatalities typically arise from renal, cardiac, or respiratory failure (Luks et al, 2003).

In the biphasic illness the initial acute or septicemic phase is characterized by bacteremia that typically lasts about one week. Most of the cases present with a febrile illness of sudden onset. Fever, chills, headache, severe myalgia, conjunctival suffusion, anorexia, nausea, vomiting, and prostration usually characterize acute leptospirosis. A substantial proportion of people infected by *Leptospira* may have subclinical disease or very mild symptoms, and do not seek medical attention (Bharti et al, 2003). In this leptospiremic phase, leptospires can be found in the blood and cerebrospinal fluid (Martinez et al, 2000). The resolution of symptoms may coincide with the second or immune phase, when circulating immunoglobulin M (IgM) antibodies begin to be produced, accompanied by excretion of spirochetes in the urine. However, fever may recur after a remission of 3 to 4 days, producing a biphasic illness.

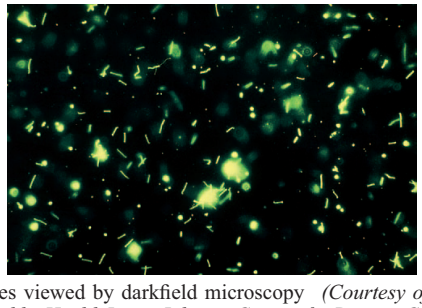
Weil's disease represents only the most severe form of the illness. This syndrome can develop after the acute phase as the second phase of a biphasic illness, or can simply present as a single, progressive illness. It is characterized by high fever, intense jaundice, bleeding, renal and pulmonary dysfunction, neurologic alterations, and cardiovascular collapse, with a variable clinical course (Vinetz et al, 1996, Carvalho & Bethle, 2002).

It has been recognized in India since 1931 (Sambasiva et al, 2003). It is especially rampant in southern, central, eastern and western India, where heavy monsoon, animal rearing practices, unplanned urbanization and agrarian way of life predispose to this infection (Sambasiva et al, 2003, Sehgal , 2006, Anagnani et al, 2003, Swapna et al, 2006,).Early and accurate diagnosis of leptospirosis is important for proper and prompt treatment, which is life saving for patients with severe illness. Leptospirosis may be confused with malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, tyhoid fever, melidiosis and others. The available methods for diagnosis include Dark ground microscopy, in- vitro culture, serology and molecular method, each one with its merits and de- merits.

Since leptospirosis is a disease of protean clinical manifestations, this disease can clinically only be suspected not confirmed. This study if implemented will help the clinicians to confirm their diagnosis of leptospirosis among patients of pyrexia of unknown origin and pyrexia with jaundice. As the treatment for leptospirosis is simple i.e. with Penicillin, patients will be benefited to the extent that administration of unwanted antibiotics can be avoided. The study will help the clinicians to suspect and diagnose this disease in a better way and thereby help the patients in general.

AIMS AND OBJECTIVE

- To find out the prevalence of leptospiral infection in patients of pyrexia of unknown origin by immunological and molecular techniques.
- To find out the prevalence of leptospiral infection in patients of pyrexia with jaundice by immunological and molecular techniques.
- Comparative evaluation of immunological and molecular techniques for diagnosis and confirmation.



Leptospires viewed by darkfield microscopy *(Courtesy of Mildred Galton, Public Health Image Library, Centers for Disease Control and Prevention*

MATERIAL & METHODS

Present study was conducted at pathology department of G.R. Medical College, Gwalior in collaboration with department of microbiology DRDE (Defence Research Development Establishment) Gwalior, from October 2011 to October 2012. In this study 77 urban – rural patients coming to J A group of hospital Gwalior for pyrexia of unknown origins and pyrexia with jaundice were selected for leptospiral infection (Gwalior group) Another group of 27 patients

were selected from flood affected rural patients from Betul district of M.P. third group of 6 patients were taken from rural flood affected areas from Banmore District Gwalior. Since Gwalior city is not a flood affected area therefore patients from flood affected area of Betul and Banmore were taken in to this study.

Inclusion criteria:
1. Sample from pyrexia of unknown origin patients.
2. Samples from pyrexia with jaundice patients

Consent:
Written and informed consent from patient, patient's parents (in case of minor) or patient's relatives was taken.

Collection and storage of samples:
All patients who were suffering from pyrexia of unknown origin and pyrexia with jaundice were taken into consideration. 5ml of blood was collected in properly labelled EDTA sterile vial by using all aseptic precautions. Samples were kept at +4°C to +8°C for brief storage and transported to the laboratory under cold condition.

Hematological tests performed were hemoglobin estimation, RBC count, total and differential count, platelet count. All these tests were done by fully automated hematoanalyser.

RBC count, WBC total count, differential count, platelet count were based on light scattering and impedance method while hemoglobin was based on cyanmethhaemoglobin method.

Peripheral blood examination
Reagents:-
1) Leishman's stain: 0.15 gm of powdered stain in 100 ml of (acetone free) methyl alcohol.
2) Buffer (pH 7.0): already prepared (Ranbaxy).

Procedure:
1) Peripheral blood smear was made on glass slide with the help of spreader slide and smear was allowed to dry.
2) Smear was covered with 10-15 drops of Leishman's stain and wait for 1 minute.
3) Equal number of drops of buffer solution was added and wait for 10 minutes.
4) Smear was washed with tap water and allowed to dry and then was observed under low power, high power and oil immersion and hundred leucocytes were counted.

ESR determination (By Wintrobe's method):
1) EDTA anticoagulated blood was filled in Wintrobe's tube to the zero mark by using Pasteur pipette and tube was placed in vertical position in the stand for 1 hour.
2) Level of erythrocyte column in terms of mm after first hour was noted at the end of 1 hour.

BACTERIAL CULTURES
***Leptospira* Serovars**
Pathogenic serovars of *Leptospira* species, viz, *L. interrogans* serovars Australis, Autumnalis, Canicola, Icterohaemorrhagiae Ictero haemorrhagiae and L. *kirschneri* serovar Grippotyphosa; maintained in DRDE, Gwalior were used in the study.

ISOLATION OF LEPTOSPIRES:
A drop of blood approximately 30 µl was inoculated into 5ml EMJH medium and further 10-fold dilution were also made. The tubes were incubated at 28-30ºC for 4-6 weeks. Cultures were examined using DFM at intervals for upto 6 weeks.

Detection of specific leptospiral DNA by PCR:
(i) Genomic DNA preparation from Bacterial Cultures
For extraction of genomic DNA from all the standard serovars of *Leptospira* species mentioned above, exponentially growing cultures were harvested at 12000 x g for 30 min, then the cells were washed with sterile PBS and DNA was then extracted with Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer's instruction. Briefly, the cells were harvested by centrifuging at 13,000 rpm for 30 min. Supernatant was discarded and the pellet was resuspended in 600 µl of nucleic acid lysis solution. Then it was incubated at 80° C for 15 min in water bath. RNAase solution (3 µl) was later added to the sample and incubated at 37° C for 30 min. Following this, 200 µl of protein precipitation solution was added and mixed. The sample was then cooled on ice for 10 min. Centrifugation was then carried out at 13000 rpm for 5 min and the supernatant was transferred to fresh sterile eppendroff containing 600 µl of isopropanol. It was then mixed gently and again centrifuged at the same speed for 15 min. After carefully discarding the supernatant, the pellet was washed with 600 µl of 70% ethanol by centrifugation. Then ethanol was aspirated and the pellet was air-dried. Finally, the DNA was rehydrated with 100 µl of DNA rehydration solution and kept for dissolving at 4° C for overnight. The DNA was later stored at -20° C for further studies.

(ii) Genomic DNA preparation from Blood Samples
Genomic DNA from blood samples was prepared using the commercially available kit (Qiagen, Germany) as per the manufacturer's instruction. Briefly, to each 200 µl of blood sample proteinase K solution (20 µl) was added. Then 200 µl of buffer AL was added and mixed by pulse-vortex for 15 sec. The mixture was incubated at 56°C for 10 min. Then 200 µl absolute ethanol was added, followed by centrifugation at 12,000 rpm for one min at room temperature. The supernatant was applied to each QIA amp spin column and the column was placed in a 2 ml collection micro-centrifuge tube and centrifuged at 8,000 rpm for one min. The tube containing filtrate was discarded. The column material was washed two times (500 µl each) with the washing buffer (AW1 and AW2 buffer) sequentially. Following the wash with AW2 buffer, the tube was spun down at 13,000 rpm for 3 min. Finally, the spin column was placed in a new sterile micro-centrifuge tube and the DNA was eluted from spin column with 50 µl of AE buffer provided in the kit. The purity of the DNA was checked in submarine gel electrophoresis using 0.8 percent agarose and TBE buffer (pH 7.5) and stored at -20° C for further use.

(iii) PCR
PCR was performed as per the method of Mullis and Faloona (1987) and Saiki and et al., 1988 using Fermentas PCR reagents. PCR amplification was performed with pathogenic serovar specific primers 16S rRNA gene (Hookey 1992) using the following steps: denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extention at 72°C for 2 min, 35 cycles, followed by 10 mins extention at 72 °C . Each 25 µl PCR reaction contained 2.5 mM $MgCl_2$, 200uM dNTPs, 50mM KCl, 10 mM tris-HCl,1 unit of *Taq* DNA polymerase, 5p moles of primers and 30ng of genomic DNA. PCR programme was run in DNA thermal cycler (iCycler, BioRad, USA). The amplified products were detected by 0.8% agarose gel electrophoresis following ethidium bromide staining.

Detection of specific antibodies in serum samples by plate- ELISA:
Indirect ELISA was performed employing recombinant Flagellar B antigen on 110 human patients.

Procedure:
Briefly, for this ELISA, 100 µl of recombinant Flagellar B antigen at a concentration of ten microgram per milliliter was coated on Microtitre ELISA plates (Maxisorp, NUNC, Germany). The plates were kept overnight at refrigeration temperature for efficient coating. The plates were blocked with three percent bovine serum albumin (BSA) under refrigeration conditions overnight. After rinsing with PBS, the plates were incubated with 1:100 dilutions of samples in PBS for one hr at room temperature. After washing thrice with PBS-Tween (PBST) and one washing with PBS, the plates were incubated with anti-human IgG/ anti-human IgM HRP conjugate (Dakopats Germany) at 1:1000 dilution in PBS for one hr at 37 C. After three washings in PBST and one washing in PBS, the reaction was developed with orthophenyldiamine (OPD)-H_2O_2 substrate solution in citrate phosphate buffer. Reaction was stopped by adding 20 µl of 2.5 M sulphuric acid. The reaction can then be read visually against a white background and recorded as clear or +, ++, or +++ reaction.

Detection of specific antibodies in serum samples by Microscopic agglutination test (MAT):
The MAT was performed on the serum samples with five serovars *L. interrogans* serovars Australis, Autumnalis, Canicola, Ictero haemorrhagiae Icterohaemorrhagiae and L. *kirschneri* serovar Grippotyphosa as per the standard procedure in microtitre plates.

Procedure:
1. Serum samples and PBS pH 7.2 bought to room temperature.

2. 50 µl of 1: 50 diluted serum taken in the 1st well of each row.25 Fl of PBS is added to all other wells. 25 µl from the 1st row transferred to the second well and so on to prepare doubling dilutions from 1:50 to 1:1600.One well each with 25 µl of negative and positive controls included.
3. 25 µl of the antigen is added to each well including those with negative and positive controls. One of the wells includes only the antigen and serves as the antigen control. The final dilutions after adding the antigen will be from 1:100 to 1 :3200. Antigen and sera are allowed to mix by gently rotating the plate on the table.
4. Microtitre plate incubated at 370C for 2 hrs.
5. Following incubation 10 µl of the mixture from the antigen control, positive control and negative controls viewed on a glass slide under low power using dark ground microscope.
6. If positive sera show clumping & this is not seen in the antigen and negative control, test samples can be read.

Interpretation:

1. The highest serum dilution showing approximately 50% agglutinated leptospires is taken as positive.
2. If agglutination with more than one serovar is seen then the serovar giving highest titre taken as infective serovar.

RESULT & OBSERVATIONS

Overall in total 110 patients were studied. Out of total 110 cases 65 (59%) were males and 45(41%) were females patients.

Table – 1 Showing haematological patterns of total 110 cases of Gwalior, Betul and Banmore groups

Total	Hb%			TLC (/mm3)		DLC(%)		Platlets count (lac/mm3)		ESR(mm after 1Hr)	
	<10gm %	10-12gm%	>12gm %	Normal (4000-11000)	Increased (>11000)	Normal DLC (Neutrophil 40-75and Lymphocyte 22-45)	Neutrophil (>75)	Normal (1.5-4.5)	Decreased (<1.5)	Normal (0-9 males,0-20 females)	Increased (>9 in males, >20 females)
Total cases (110)	35	57	18	98	12	95	15	73	37	76	34
(100%)	31.9%	51.8%	16.3%	89.1%	10.9%	86.4%	13.6%	63.3%	33.7%	69.1%	30.9%

Table - 2: Showing patient of pyrexia of unknown origin and patients of pyrexia with jaundice in Gwalior, Betul and Banmore Groups

S No.	Group of patients	No of pyrexia of unknown origin (PUO) patients		No of pyrexia with jaundice patients	
		Number	%	Number	%
1	Gwalior group 77 cases	34	44.15%	43	55.85%
2	Betul group 27 cases	23	85.18%	04	14.82%
3	Banmore group 06 cases	06	100%	00	0%
4	Overall total 110 cases	63	57.3%	47	42.7%

Table - 3: Showing result of culture and Polymerase Chain Reaction(PCR) in all three groups

SNo.	Name of group	Culture		Polymerase chain reaction(PCR test)	
		Positive	Negative	Positive	Negative
1	Gwalior group (77 cases)	0(0%)	77(100%)	-	-
2	Betul group (27 cases)	0(0%)	27(100%)	0(0%)	20 Tested cases(100%)
3	Banmore group (6 cases)	0(0%)	6(100%)	-	-
4	Overall three groups total 110 cases	0(0%)	110(100%)	0(0%)	20Tested cases(100%) Betul group

Table - 4: Results of serum IgM, IgG and combined IgM and IgG in all three groups

S No.	Name of group	Serum IgM antibody				Serum IgG antibody				Combined IgM/IgG antibody			
		PUO cases		Pyrexia with jaundice		PUO cases		Pyrexia with jaundice		PUO cases		Pyrexia with jaundice	
		Total	+ve	Total	+ve	Total	+ve	Total	+ve	Total	+ve	Total	+ve
1	Gwalior group (77)	34	3 (8.82%)	43	4 (9.30%)	34	14 (41.2%)	43	18 (41.86%)	34	3 (8.82%)	43	4 (9.3%)
2	Betul group (27)	23	10 (43.47%)	4	1 (25%)	23	7 (30.44%)	4	0 (0%)	23	4 (17.4%)	4	0 (0%)
3	Banmore group (06)	6	3 (50%)	0	0 (0%)	6	3 (50%)	0	0 (0%)	6	3 (50%)	0	0 (0%)
4	Overall total three groups (110)	63	16 (25.39%)	47	5 (10.64%)	63	24 (35.93%)	47	18 (38.30%)	63	10 (15.87%)	47	4 (8.51%)

Table - 5: Results of serotypes by microscopic agglutination test (MAT) in Betul group

Leptospiral serovars									
Australis		Autumnalis		Canicola		Grippotyphosa		Icterohaemorrhagiae	
Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
0 (0%)	20 (100%)	13 (65%)	7 (35%)	3 (15%)	17 (85%)	0 (0%)	20 (100%)	1 (5%)	19 (95%)

SUMMARY & CONCLUSION

Present study was conducted at pathology department of G.R. Medical College, Gwalior in collaboration with department of microbiology DRDE (Defence Research Development Establishment), Gwalior from Oct. 2011 to Oct. 2012. In this study 77 urban and rural patients from Gwalior city, 27 patients from flood affected rural area of Betul and 6 patients of Banmore were included.

- In Gwalior group out of 77 cases males were 52 (67.53%) showing male preponderance but in Betul group females were 14 out of 27(51.85%) and in Banmore group 6 out of 6 (100%) patients were females showing female preponderance. In all three groups overall out of total 110 cases 65(59%) males showing male preponderance. Possible reason for male preponderance is that males are more occupationally active and there is no sex linked susceptibility found.
- Majority of leptospirosis patients were seen in 26-45 yrs age group 57 cases out of 110 (51.4%). Least common 3 cases (2.7%) were found in <5 yrs of age. It is concluded that leptospirosis is a disease of occupationally active age group.
- In nonspecific haematological tests anaemia was found in 31.9% cases, normal TLC in 89.1% cases, neutrophilia in 13.6% cases, thrombocytopenia in 33.7% cases and increased ESR in 30.9% cases. From these findings the inference is that haematological parameters are not consistent and not diagnostic for leptospirosis.
- Leptospirosis culture tests were negative in total 110 cases (100%). PCR test was conducted in only 20 cases of Betul group out of 27 cases and all 20 cases (100%) were negative. It was concluded that it is hard to find leptospires in blood after 5 to 7 days, therefore culture not recommended for late phase of disease.

Culture is also tedious, complicated, expensive, technically demanding and time consuming and even after that rarely positive, so not a preferred diagnostic method but positive results are always confirmatory. Like wise though PCR is very sensitive direct confirmatory test but only in early stages (1 to 5 days) of disease but late collected samples are negative, due to absence of leptospiral DNA.

- In serological tests IgM positivity was found in 3 out of 34 (8.82%) cases of PUO in Gwalior group while 4 out of 43 (9.30%) cases were seen in pyrexia with jaundice group. Serum IgG was 41.2% in PUO group while 41.86% in pyrexia with jaundice group, combined IgM and IgG were found in 8.8% in PUO group and 9.3% in pyrexia with jaundice group. In Betul group IgM positivity was found in 43.47% in PUO group and 25% in pyrexia with jaundice patients. Serum IgG positivtiy was found in 30.44% cases of PUO and jaundice with pyrexia shows 0% cases, combined IgM/IgG antibodies were found in 17.4% cases in PUO but 0% cases in jaundice patients. In Banmore group 50% IgM positivity was seen in PUO group while 0% incidence in jaundice group. Serum IgG was 50% positive in PUO group while jaundice group showed 0%. The combined IgG/IgM positivity seen in 50% cases in PUO and 0% in pyrexia with jaundice group.
- IgM antibodies detectable from 6th to 10th day of disease and reach peak level in 3 to 4 wks. IgM detection is best diagnostic method though not a gold standard test, for lepstospirosis demonstration of rising titer of IgM antibody in paired sera are diagnostic.
- For recent infection IgM antibody is rapid and practically simple method and IgG positivity signifies the possibility of old infection of leptospirosis.
- MAT (microscopic agglutination test) is gold standard test for diagnosis of leptospirosis. In 20 cases of Betul group leptospira serovar autumnalis was found to be the most common that is 13 cases (65%) followed by serovar canicola 3 cases (15%) and icterohaemorrhagiae 1 case (5%). Serovar australis and Grippotyphosa were not found (0%).
- MAT test is very specific gold standard test and essential for seroepidemiological studies. But this test is technically demanding and time consuming and may require large panel of serovars at times, and biohazardous as live bacteria are used as antigens. Paired sera are recommended for better result.
- Since leptospirosis is present in all ecological zones of world it should be considered as differential diagnosis of undifferentiated fever. Prevention of disease is largely dependent on sanitation measures. Integrated disease surveillance programme (IDSP) may guide the clinical and public health interventions for controlling leptospirosis.

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