A Sero-Epidemiological Study on Leptospirosis among Children

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Abstract

Background & Objectives: Leptospirosis is still posing to be a major health hazard among developing countries like India. The present work was undertaken to study the incidence of leptospirosis among children of Salem, a District in Tamilnadu, South India.

Methods: Repeat sera samples from 115 suspected leptospirosis cases (HBsAg negative) who have attended the Government General Hospital during the period from January 2009 to January 2011 were subjected for the sero-study. Microscopic agglutination test (MAT) using a battery of six live Leptospira antigens was used for the study.

Results: Among the suspected boys leptospiral seropositivity of 55.65 % and among the girls 44.34% were observed. Leptospira autumnalis, icterohaemorrhagiae and javanica were the predominating serogroups noted in this study. Male preponderance was clearly seen. Among the age group tested 6-10Yr were more affected than the age groups of 1-5 Yr and 11 to 15 Yr in both genders.

Conclusions: A number of leptospirosis outbreaks have occurred in the past few years in various places all over the world and in India. The precise identification and classification of leptospires is important for epidemiological and public health surveillance especially in children.

Key words: Children, Zoonosis, Leptospirosis, Leptospira, Microscopic Agglutination Test (MAT).

Introduction

Leptospirosis is a zoonosis that occurs throughout the world especially to tropical and subtropical regions (1). Leptospirosis is a reemerging disease in India (2). Leptospirosis is more common in southern parts of India and large number of outbreaks has been noticed during the period of October to December, every year in Tamilnadu (3&4). For the first time in India, serovar javanica has been isolated from a human clinical case at Chennai (5). Leptospirosis is believed to be an important cause of asptic meningitis (6) and acute febrile illness in children. Young children are often exposed to leptospira because of their playful nature like swimming in lakes, fishing, etc and from petting animals and exposure to direct or indirect contact with infected animals' urine (7). Due to its nonspecific symptoms that mimic better-known diseases, leptospirosis has been frequently undiagnosed or misdiagnosed (8). It is very important to screen all the PUO cases for leptospirosis and follow them up for the development of signs and symptoms of leptospirosis to confirm unequivocally the infection (9 & 10). However
the true incidence and prevalence of leptospirosis in children is not known exactly. The present paper aims at finding out the prevalence of leptospirosis among children in the Salem District of Tamil Nadu in India.

**Materials and Methods**

115 repeat blood samples collected from clinically suspected (HBsAg negative) hepatitis cases of leptospirosis admitted to Government general Hospital Salem during January 2009 to January 2011 were subjected for this sero study. About 5 ml of the whole blood sample were received within two hours and the sera was separated and preserved at -20°C until use. Microscopic Agglutination Test (MAT) was performed on the samples using six live leptospiral strains as antigens. The pathogenic strains belonged to the serogroups: *L. australis*, *L. autumnalis*, *L. icterohaemorrhagiae*, *L. canicola*, *L. javanica* and the non pathogenic (genus specific) belonged to strain *L. semaranga* (Patoc I). The antigens used were 5–7 days old autoagglutination-free cultures grown in Elinghausen McCullough Johnson and Harris (EMJH) liquid medium (Difco, Sparks, MD) with approximately $1 \times 10^8$ to $2 \times 10^8$ organisms/mL. The assay was performed by a modified Galton technique. With six serovars of leptospira used as antigen. Briefly 25 µl of sera were diluted to 1:50 with Phosphate Buffer Saline (PBS) and added into six wells of micro titer plate. 25 µl of each leptospire serovars was added into each well. The specimens were mixed gently. After leaving for 2 to 3 hours at room temperature, 3 µl of the suspension was dropped on a slide. The agglutination was absorbed under dark field microscope at a final magnification of 100X. Sera showing positive reaction were then retested against the respective serovar using doubling dilutions starting from 1:20 up to end titers. A titer of 1: 80 or above was considered as positive (11).

**Results**

Of the 115 blood samples (64 boys and 51 girls), 71 samples showed positive result for leptospirosis by MAT, with an overall sero positivity of 61.73 percent. The various serovars identified were *L. autumnalis* (64.78 percent), *L. icterohaemorrhagiae* (15.49 percent), *L. javanica* (9.85 percent), *L. canicola* (7.04 percent), and *L. australis* (4.86 percent) (Table 1).

**Table 1 Sero Positive cases of children with suspected leptospirosis**

<table>
<thead>
<tr>
<th>Live Antigen</th>
<th>Male Positive</th>
<th>Female positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. autumnalis</em></td>
<td>28 (64.78%)</td>
<td>18 (43.90%)</td>
</tr>
<tr>
<td><em>L. australis</em></td>
<td>1 (4.86%)</td>
<td>1 (2.43%)</td>
</tr>
<tr>
<td><em>L. canicola</em></td>
<td>2 (7.04%)</td>
<td>2 (7.31%)</td>
</tr>
<tr>
<td><em>L. javanica</em></td>
<td>4 (9.85%)</td>
<td>4 (7.31%)</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em></td>
<td>6 (15.49%)</td>
<td>5 (12.19%)</td>
</tr>
</tbody>
</table>

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Table 2: Age and Sex wise distribution of leptospirosis among Salem children

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total Cases</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
</tr>
<tr>
<td>0-5</td>
<td>22</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>(55%)</td>
<td>(45%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>6-10</td>
<td>26</td>
<td>24</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>(52%)</td>
<td>(48%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>11-15</td>
<td>16</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>(64%)</td>
<td>(36%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

The overall seropositivity of children in the age group 0-5 years, 5-10 years and 11-15 years was seen to be 57.50 percent, 66 percent and 60 percent respectively. In boys, the seropositivity was 59.09 percent, 69.25 percent and 62.5 percent respectively and in girls it was 55.55 percent, 62.5 and 55.5 percent respectively (Table 2 for absolute data).

Discussion

Earlier studies performed in Tamil Nadu, based on isolation and serology among human beings have shown that L. autumnalis was the dominant infecting sero group (12), which is true according to the present survey. L. autumnalis predominance was also reported in Chennai by Saravanann et al. (13). The exact seropositivity rates for leptospirosis in Indian children are not known, though periodic outbreaks are known to occur. Although the study has been conducted exclusively among children, it proves the prevalence of leptospirosis being common not only in working age population (15-59) but also among children. According to Natrajssreenivasan (14) leptospirosis is not commonly reported in Salem perhaps because of the difficulty in making clinical diagnosis and the leptospira cases might have been overlooked by the clinicians. The present empirical data collected from Salem district supports Natrajssreenivasan’s opinion that the area is endemic for the disease. In India, leptospirosis is endemic in most of the urban areas (15). The present study indicates the endemic prevalence of the disease in rural areas also. The high rate of prevalence in the age group 5-10 indicates their frequent exposure to infected animal’s urine contaminated due to rodents, cattle, dogs, cats, pigs etc., reared in the surroundings for the transmission of the bacteria.

Conclusion

Health and hygiene are important wealth of not only individuals but are real indicators of human development of a country. The prevalence of leptospirosis is common among working population in foreign countries like Italy. But, in India, the prevalence of leptospirosis among children is a matter of great concern. Adequate attention and care has to be provided for the healthy life of children in our society. Children should not walk and play without protective clothing’s in stagnant water. Important measures of prevention of such diseases are through rodent control and avoidance of contact with contaminated water and soil and immunization of livestock. The present study is only an initial investigation and more light has to be thrown on the different diagnostic techniques for the early detection of leptospirosis to atleast various populations of Salem district.
References


Effect of *Phyllanthus amarus* extract on SphH gene of *Leptospira autumnalis* studied by an in-house PCR

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**Abstract:** Leptospirosis is an important re-emerging infectious disease in human resulting from direct or indirect contact with the infected animals’ urine. Pathogenic *Leptospira* requires ions for their growth and these spirochetes use their hemolysins such as the SphHingomyelinases to obtain ions from host red blood cells during their infection which results in anemia. Due to the side effects encountered in most cases of chemotherapy today, people around the globe show more interest in alternative medicines especially from herbal sources. The present study was designed to evaluate the effect of *Phyllanthus amarus* extract on SphH gene of *Leptospira autumnalis* using an in-house PCR. During the observation of the amplified products from the reaction mixture, the *L. autumnalis*, which were treated with the plant extract, were not showing the specific DNA band as that of the negative control (triple distilled water). Interestingly the positive control samples (extract untreated *L. autumnalis*) evinced specific DNA band with respect to the SphH gene amplification.

**Keywords:** *Leptospira autumnalis*, SphH gene, *Phyllanthus amarus*, PCR

**Introduction**
Leptospirosis is considered as an important re-emerging infectious disease worldwide [1]. The disease in human results from direct or indirect contacts with the infected animal’s urine. The pathogenic *Leptospira* cause diverse damage in humans due to the virulence factors produced by the bacteria and among them the hemolysins are the most important virulence factor. Hemolysin

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SphH (sphingomyelinases) gene has been reported in most of the Leptospiral serovars. Pathogenic Leptospira requires ions for their growth and these spirochetes probably use their hemolysins such as the sphingomyelinases to obtain ions from host red blood cells during infection which results in erythrocyte lysis in host [2]. Doxycycline may be used to prevent infection but it shows some side effects such as diarrhea or loose stools, nausea, abdominal pain, vomiting and may cause tooth discoloration if used in person below 8 years of age. Penicillin may cause a temporary exacerbation of the symptoms [3]. Due to the side effects produced by the chemotherapy today, people around the globe are showing growing interest and preference to alternative medicines especially of herbal extracts. An effective course of treating leptospirosis still remains unsolved problem. To overcome the adverse reaction by the above drugs, herbal-based therapeutics had been used in treating leptospirosis [4]. *Phyllanthus amarus* (L.) belongs to the family *Euphorbiaceae* commonly called as Bahupatra in India. Traditionally, these plants are ayurvedic herbs used in southern India for the treatment of liver diseases [5]. Studies on the herbal extracts effect on molecular level damage of *Leptospira* are scanty. In view of these drawbacks and to emphasize the importance of alternative medicine for this dangerous disease this research work was designed to evaluate the effect of *P. amarus* extract on SphH gene of *Leptospira autumnalis* using an in-house PCR.

**Materials and Methods**

**Leptospira Culture**

*Leptospira autumnalis* cultures in EMJH semisolid medium (Difco, Sparks, UK) were procured from the WHO Center for Reference and Research on leptospirosis, Brisbane, Australia.

**Collection of Plant material**

The plant *Phyllanthus amarus* were collected from ABS medicinal plant research center, Karippatti, Salem, Tamilnadu, India during the month of December 2011. Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles.

**Preparation of aqueous extract**

Ten grams of air-dried powder was added to distilled water and boiled on slow heat for 2 h [6]. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume one-fourth of the original volume [7].

**Leptospira autumnalis DNA extraction**

1ml of *L. autumnalis* culture was exposed with 50μl of *P. amarus* extract and incubated at room temperature for 24 h [8]. In the positive control vials instead of *P. amarus* extract 50μl sterile triple
distilled water was added. After incubation period the respective DNA samples were isolated from both the set of vials by using the QIAamp DNA extraction kit (QIAGEN, Hilden, Germany).

**Amplification of Leptospira autumnalis SphH gene**

The in-house PCR was attempted using the following primers for the amplification of SphH gene [9]:

5'- GGCTCGAGATGCGAAACATTTCCGAAA-3' (Forward)

5'- CCAGATCTTCGACTTTAGGATCGTTAT-3' (Reverse)

In a sterile 0.5ml microfuge tube, the following contents were added: 4μl of 10X PCR buffer, 7μl of Template DNA, 1 μl of forward and reverse primer, 2μl of 25 mM magnesium chloride, 4μl of dNTPs, 2μl of Taq DNA polymerase and 29μl of triple distilled water. The tubes were vortexed gently and spun the tubes for ten seconds to settle the contents and the tubes were placed in a controlled temperature heat block of the thermocycler. The thermal profiler involved 30 cycles of each of the following: Denaturation at 94°C for 1 min 30 sec, Primer annealing at 56°C for 1 min and Polymerization at 72°C for 1 min. In the final step, the set up was kept at 72°C for 3 min extension to ensure the complete polymerization [10]. After the amplification the PCR products were taken in gel loading buffer and run by electrophoresis in 1.5% agarose gel along with the untreated leptospiral DNA as positive control and plain triple distilled water as the negative control. The DNA bands were visualized and documented (BIO-RAD, USA) [11].

![Amplification of SphH gene of Leptospira autumnalis](image)

**Fig. 1** Amplification of SphH gene of *Leptospira autumnalis*
Results
Polymerase chain reaction of the \textit{P. amarus} extract treated and untreated (positive control) leptospiral DNA along with the sterile plain triple distilled water (negative control) yielded interesting results. The positive and negative controls were consistently giving concordant results by the in-house PCR for more than five repetitions. The extract treated leptospiral DNA template did not amplify for the \textit{SphH} gene in spite of eight repeated attempts. Successful demonstration of the specific DNA band of approximately 1.6 kbp in the positive control indicated the reliability of the standardized in-house PCR. The damage caused by the extract of \textit{P. amarus} on the leptospiral DNA prevented its amplification and the result was similar to that of the negative control (Fig. 1). Thus the absence of DNA band of leptospiral DNA on the agarose gel indicated that the active principle of \textit{P. amarus} was effective in damaging the \textit{SphH} (sphingomyelinase) gene.

Discussion
The available therapies for leptospirosis in modern medicine are very limited, potential alternatives from traditional medicine and their respective mechanisms of the action are worth investigating [12]. Recently, significant attention has been focused on plant extracts and biologically active compounds isolated from popular plant species [13]. However, the effect active principle of of plant extracts on the pathogen’s at molecular level is poorly studied. In the present study \textit{Phyllanthus amarus} was observed to be effectively damaging the leptospiral DNA. Therefore the template DNA could not give any amplification when targeted for this most virulent gene (\textit{SphH}).

An earlier study revealed antileptospiral effect of \textit{P. amarus} and \textit{Eclipta alba} against \textit{L. icterohaemorrhagiae}. In their study both aqueous and methanol extracts at the concentration of 1\mu g/ml was reported to cleave \textit{Leptospira} DNA completely [14]. The complete DNA damage in the present study this phenomenon of complete DNA damage could be attributed for the failure in the amplification of the \textit{SphH} gene in spite of many repeated trials. Indian medicinal plants such as \textit{Plectranthus amboinicus} extracts are reported to be not only hapato-renal protective but also they did possess anti-leptospiral effects [8]. Another herbal extract study also reported antileptospiral activity of \textit{Eclipta alba} when tested by both tube dilution and micro dilution technique [15]. An in-vitro experiment conducted using \textit{Zingiber officinale} (Ginger), \textit{ Ocimum sanctum} (Tulasi) and \textit{Piper nigrum} (Black pepper) against various \textit{Leptospira} serogroups like \textit{L. australis}, \textit{L. pomona}, \textit{L. autumnalis}, \textit{L. icterohaemorrhagiae}, \textit{L. canicola}, \textit{L. copenhagenii} and \textit{L. semaranga} had revealed that except \textit{L. autumnalis} all the other serogroups were susceptible to \textit{Ocimum sanctum} (Tulasi) and \textit{Piper nigrum} (Black pepper). Further, the \textit{L. autumnalis} a most dominant serovar especially in west Chennai was susceptible only to the extracts of \textit{Z. officinale} [16].

The present PCR study has an advantage of utilizing the tool for more accurate diagnosis of leptospirosis this is in full agreement with a previous study stating that the cross reactions caused by exposure to leptospires of the same group can occur for example infection by \textit{L. balcanica} and \textit{L. medenensis} can produce false positive \textit{L. hardjo} reactions. This technique was earlier used by Truccolo \textit{et al.} [17] with a single set of arbitrary primers in quantitative PCR to evaluate the...
ampicillin, afloxicin and doxycycline for the treatment of experimental leptospirosis. The study is in full agreement with the present PCR study. Therefore the present study suggests that the use of *Phyllanthus amarus* could play a vital role in covering the basic health needs in developing countries and could offer a new source of herbal medicine for leptospirosis.

References


RESEARCH ARTICLE

SDS- PAGE STUDY ON LEPTOSPIRA AUTUMNALIS PROTEIN DAMAGE DUE TO PHYLLANTHUS AMARUS AND ECLIPTA ALBA

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ABSTRACT

Leptospirosis is a potentially fatal infectious disease which is neglected and very widely prevalent in India causing a major public health problem. The present leptosporial research is focused on Leptospira autumnalis protein damage due to Phyllanthus amarus and Eclipta alba. SDS – PAGE study on Leptospira autumnalis protein damage by both extract treated sample damaged 65 kDa region, a major outer protein (Sphingomyelinsase). Interestingly Phyllanthus amarus treated leptosporial protein additionally damaged 25 kDa region, inner membrane protein. Protein damage were reported at various levels in Phyllanthus amarus than Eclipta alba. Further in-depth analysis of phytochemical plant compounds against the leptosporal proteins will reduce the side effects produced by synthetic drugs.

INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic spirochaetes of the genus Leptospira (Fentahun and Alemayehu, 2012). The organisms are maintained in nature by chronic renal infection of carrier mammals, which excrete the organisms in their urine. Humans become infected through direct exposure to infected animals or their urine or through indirect contact via contaminated water or soil (Kamath and Joshi, 2003). The adhesion of Leptospira to host tissue components is thought of as an initial and necessary step for infection and pathogenesis. Attachment to host cells and ECM components is likely to be necessary for the ability of Leptospira to penetrate, disseminate and persist in mammalian host tissues (Breiner et al., 2009). In Silico analysis and experimental techniques employed to identify leptosporial surface-exposed proteins that might have potential roles in Leptospira adhesion and pathogenesis (Pinne and Haake, 2009). It is known that Leptospira can alter their biosynthetic mechanism for the production of the lipopolysaccharides in their outer membrane thus allowing them to adapt to new hosts (Nascimento et al., 2004). Penicillin, cephalosporins, tetracyclines and macrolides have been widely used in the treatment of human leptospirosis. However, when these antimicrobial agents are used for the treatment of leptospirosis, long-term therapy with large doses may be required from the early stage of the disease until the appearance of antibodies (Misao et al., 1955). Due to side effects produced by chemotherapy today, people are showing greater interest towards herbal medicines. It is safer than synthetic medicines because the phytochemicals in the plant extract target the biochemical pathway. To overcome the side effects produced by synthetic drugs, herbal based therapeutics had been used in treating leptospirosis (Emmanuelleides et al., 1994).

Phyllanthus amarus (L.) belongs to the family Euphorbiaceae commonly called as Bahupatra in India. Traditionally, these plants are ayurvedic herb used in southern India for the treatment of liver diseases (Portio et al., 2001). Eclipta alba belongs to the family Asteraceae grows in tropical and subtropical countries. It is commonly known as Karshalenganni in Tamil (Suresh Kumar et al., 2005). Traditionally, these plants are ayurvedic herb used in southern India for the treatment of liver related diseases (Nirmaladevi and Periyanyagam, 2011). The herb contains wederolactone as coumarin derivatives, the first one has been responsible as major anti-hepatoxetic compounds of this plant. It is an active ingredient of many herbal formulations prescribed for liver ailments and shows effect on liver cell generation (Portio et al., 2011). Herbal extract researches on protein damage of Leptospira autumnalis are not documented. Keeping these drawbacks the current research work was designed as SDS-PAGE study on Leptospira autumnalis protein damage due to Phyllanthus amarus and Eclipta alba.

MATERIALS AND METHODS

Collection of Plant material

The commonly available medicinal plants of our region P. amarus and E. alba were received from ABS medicinal plant research center, Karippatti, Salem Tamilnadu, India during the month of December 2011. Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles.
Preparation of aqueous extract

Ten grams of air-dried powder was added to distilled water and boiled on slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume one-fourth of the original volume (Sadique et al., 1989).

Effect of Phyllanthus amarus and Eclipta alba on Leptospira autumnalis protein damage

1ml of Leptospira autumnalis culture was treated with 50μl of plant extracts and incubated at room temperature for 24 hours (Nirmaladevi and Periyarayagam, 2011). The treated samples were further analyzed for the protein damage by SDS PAGE.

Leptospira Culture

Leptospira autumnalis cultures in EMJH semisolid medium (Difco, Sparks, UK) were received from the WHO Center for Reference and Research on leptospirosis, Brisbane, Australia.

Whole cell lysate antigen

The above mentioned medium with the bacterial growth was used for lysis. The whole cell culture with and without exposure to the chosen herb extracts were sonicated in a chilled atmosphere with the frequency of 20Hz3 times for a duration of 15seconds giving a few seconds interval. This sonicated culture was centrifuged at 4°C, in a refrigerated centrifuge with a revolution of 10,000 rpm for 20mts. The pellet was reconstituted in a very little quantity of Tris Hcl buffer, pH6.8. The supernatant was added with equal amount of 10% TCA and centrifuged at a gyration of 2000rpm for 10minutes. The pellet was given 5 to 6 times petroleum ether wash and was reconstituted with very little quantity of the above mentioned buffer. The protein samples were cryopreserved until use (Biswas et al.,2005).

Estimation of antigenic protein

The partially purified leptosomal whole cell lysate protein was subjected for protein estimation study following the standard procedure (Lowry et al., 1951).

SDS-PAGE

Preparation of whole-cell lysate proteins was performed by using the following lysis solution: 60 mM Tris-HCl (pH 6.8)-10% glycerol-2% SDS-5% mercaptoethanol-0.1% bromophenol blue. Whole cell lysate proteins were boiled for 10 min, respectively, in the lysar buffer. Discontinuous SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970) in a vertical electrophoresis apparatus (Biotech, Yercaud) with some modifications. Resolving gel used was 12 per cent acrylamide over hanged with 5 per cent of stacking gel. Twenty micrograms of whole-cell protein lysate of detergent-soluble protein of the strain prepared in 2X gel loading buffer was added to the appropriate well along with molecular weight marker. The samples were first run at a constant voltage of 50 V, till the dye front crossed the stacking gel. Then the voltage was increased to 100V till the dye front reached the end of the gel. Thereafter, the gel was subjected to the coomassie brilliant blue R-250 (Sigma, USA) staining for 1 h and then destained for 6-8 h with several changes of destaining solution.

RESULT

The interesting results of SDS-PAGE study on P. amarus and E. alba exposed L. autumnalis protein profile are as follows: Some deeply stained and faintly stained protein bands were observed during the analysis. Low molecular weight protein bands observed were 22, 25, 41 and 45 kDa. These protein bands were almost intact in both the control and E. alba treated samples. A deeply stained 65 kDa band which was reported to be a major immunogenic protein was intact in the control (untreated Leptospira). The present study gives us hope in the management of Indian Leptospirosis. Thus in spite of many repeated trials the extracts treated Leptospira was evincing sphingomyelinase protein damaged.

Proteins damage were noted at higher molecular weight regions namely 94 and 105.5 kDa of both extract treated samples however, no such damage was observed for the untreated (control) Leptospira protein samples. The major protein band at 65 kDa region which was reported as Sphingomyelinase protein was clearly resolved in the present SDS – PAGE study using the bacterial whole cell lysate antigens. (Fig. 1). The virulent protein was found intact in the control however, a mild damage in E. alba exposed Leptospira and a clear damage in P. amarus exposed Leptospira was very interesting to be noted. Moreover the other important inner membrane protein damage was observed only for P. amarus at 25 kDa regions. However, the other important outer membrane protein did not show any damage at 45 kDa region in spite of the repeated exposure by both the extracts on the Leptospiral samples.

DISCUSSION

Thus successfully the major immunogenic protein at 65KDa region (Sphingomyelinase) was isolated from the leptospirosis using their whole cell lysate antigens. The Sphingomyelinase protein was found intact as that of the control and showing damage by the P. amarus and E. alba treated leptospiros. It was suggested that the SPH as a novel leptospirosis haemolysin identified as a pore-forming protein among the pathogenic leptospiros. Direct membrane damage of sheep erythrocyte and mammalian cells caused by the SPH in vitro studies indicated its potential role in pathogenesis of leptospirosis (Seoung et al., 2002). It was further stated that the proteins of 67, 41, 35, 32, 28 and 22 kDa were the major outer membrane protein reported in the commonly circulating leptospiros serogroups in India, while 94, 32, 25 and 18 kDa proteins were of inner membrane (Biswas et al., 2005). In the present study the inner membrane protein 25 kDa was completely damaged in P. amarus treated sample where as not damaged in E. alba treated sample. 25 kDa inner membrane
protein was designated as LfhA (Leptospiral factor H- binding protein) and also found to bind human factor H related protein 1 (FHR-1). The capacity of pathogenic leptospiroa to bind host extra cellular matrix and factor H depends on virulence property (Verma et al., 2006). In the present study those major immunoreactive proteins reported by Biswas et al., (Biswas et al., 2005), were also reported with some minor positional changes respectively at 65, 45 and 31.0 kDa regions. This could be due to serogroup difference. In the present study L. autumnalis was used but in the previous study L. pomona was used. Thus it is evident by the present study that sphingomyelinase could be demonstrated in between 66 kDa and 77 kDa region. Sphingomyelinase damage observed by the treatment of both sample is very interesting to note because the protein is in the major virulent factor of leptospiroa (Volina et al., 1986).

In the present study some minor bands were also observed at 22, 25, 41 and 45 kDa, this observation is similar to the report of Gallyanee and coworkers who have reported similar pattern of multiple reactive bands, ranging from 10 to 90 kDa (Gallyanee et al., 2007). In an earlier study it was reported that a protein with an apparent molecular weight of 47 kDa was observed but in the present study this band was reported at 45 kDa. It was included in a group of outer membrane proteins which was described in the leptospiroa of different serogroups (Cullen et al., 2002). The antigenic and genetic conservation of the two proteins OMPL1 and Lip41, were indicated and it was further stated that these could be potential candidates for the development of diagnostic test system for leptospiroa (Natarajaseenivasan et al., 2005). This present study is going hand with the above study. Paired bands were reported at 45 kDa in both extract treated samples. A novel 45 kDa OMP from L. kirschneri was reported to induce antibody response in hamsters indicating that LipL45 was expressed during infection (Matsunaga et al., 2002). An earlier study which reported the antileptospiroa activity of E. alba by both tube dilution technique and microdilution technique and the results showed showed better inhibitory action against various serogroups of Leptospira interrogans (Prabhu et al., 2008). In the present study protein damage was observed at various levels P. canicola treated sample than E. alba. The present study gives a clear insight on the Sphingomyelinase and inner membrane protein damages by evincing either distorted protein bands or absence of the bands at specific region in comparison with untreated (control) leptospiral protein bands. The herbal extracts exposed L. autumnalis after 24 hours were showing clear protein damage at 65 kDa, as observed in the slab gel protein profile.

Reference


A comparative study on microscopic agglutination test and counterimmunoelectrophoresis for early detection of human leptospirosis

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Abstract

Background and Objectives: Leptospirosis is a potentially fatal bacterial disease that mimics many diseases; therefore, laboratory confirmation is pivotal. Though microscopic agglutination test (MAT) is accepted as World Health Organisation (WHO) reference test, it has got many pitfalls such as being hazardous, tedious, cumbersome and expensive. Counterimmunoelectrophoresis (CIE) is popularly used for diagnosing many infectious diseases but rarely for Leptospirosis. The aim of this study is to find suitability of CIE for the routine laboratory diagnostic purposes.

Materials and Methods: Repeat sampling (paired sera) was possible from 401 subjects of which 181 were in-patients of Salem Government General and Private Hospitals and the remaining 220 MAT negative healthy college students gave their consent for the study. All the 802 sera samples were collected from January 2009 to November 2012 and subjected to the present study. After carrying out MAT and CIE on the suspected and control samples, a comparative evaluation was conducted. McNemars test method was used to find out the significant difference between the two tests in the early diagnosis. Result: The sensitivity, specificity, Positive Predictive value (PPV), Negative Predictive value (NPV) and Efficiency test for CIE were 96.80%, 89.28%, 95.23%, 92.59% and 94.47%, respectively. The corresponding values for MAT were 95.90%, 89.83%, 95.08%, 91.37% and 93.92%, respectively. There was no significant difference between MAT and CIE at 95% and 99% confidence intervals according to McNemars test. P value in the early stage of illness was greater for CIE than MAT when Polymerase Chain Reaction (PCR) was used as Gold Standard of diagnosis.

Interpretation and conclusion: It was concluded that the CIE could be advantageous over MAT due to its safety, rapidity, simplicity, economical and easy for large number of samples. It can answer little earlier than MAT and found as reliable as that of MAT. Since both the tests had shown similar efficacies in the later stage of the illness, the importance could be given to CIE due to early diagnosis.

Key words: Counterimmunoelectrophoresis, diagnosis, microscopic agglutination test, Salem, suspected leptospirosis, sensitivity, specificity

Introduction

Leptospirosis is a potentially fatal infectious disease, which is neglected and very widely prevalent in India causing a major public health problem. It can display a wide array of clinical symptoms mimicking better-known illnesses. It may be confused with malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, meningitis and others. Leptospirosis is underreported due to lack of clinical suspicion and barriers to diagnostic capacity for confirmation. Early diagnosis and the ability to differentiate leptospirosis from other diseases are important to reduce the risk of more serious infection or mortality.

Microscopic agglutination test (MAT) is accepted as World Health Organisation (WHO) reference test in spite of several pitfalls like being hazardous, tedious, cumbersome, requiring expensive medium for regular maintenance of culture, technically demanding etc. MAT involves serial dilutions of patient sera mixed with live suspensions of Leptospira. If agglutinating antibodies against Leptospira are present, the spirochaetes will clump. This can be observed only under dark field microscope. MAT is available only in few research institutes. It is not helpful for routine laboratory diagnosis of leptospirosis during acute illness. For these reasons, MAT is not frequently employed for diagnosis other than research. Therefore, an alternate tool, which has got good sensitivity and specificity equivalent to MAT, is needed for rapid, economic and simple diagnosis of leptospirosis.
Counterimmuno-electrophoresis (CIE) is a popular method used for the diagnosis of numerous infectious diseases but less utilised for leptospirosis diagnosis. CIE is safe, fast, easy to perform, inexpensive and ideal for analysis of large number of samples. CIE is able to detect acute infection earlier than MAT, employing only one genus specific antigen, which can be conveniently adapted for leptospirosis cases. The test gives result within 10-15 minutes. Antigen stability for CIE technique remains 6 months without loss of titer, whereas for MAT live antigens needed to be sub-cultured every week in the costly Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium. The suitability of CIE compared with MAT in leptospirosis diagnosis has not been well researched on. The aim of this study is to compare the relative sensitivity, specificity and efficiency of CIE with MAT for early detection of leptospirosis to find the suitability of CIE for laboratory diagnosis where MAT is not possible. The present study is essential for a developing country like India which has leptospirosis outbreaks during rainy season and the preponderance of simple diagnostic laboratories throughout our country.

Materials and Methods

Repeat sampling (paired sera) was possible from 401 subjects of which 181 were in-patients of Salem Government General and Private Hospitals and the remaining 220 were MAT negative healthy College students who came as volunteers for the study. All the 802 sera samples were collected from January 2009 to November 2012 after taking their written consent from their or their legal guardian (if they were minor). Suspected cases were selected based on Faine’s criteria. The clinical signs and symptoms were pyrexia, meningitis, myalgia, conjunctival suffusion, nausea, vomiting, petechial haemorrhages, rashes, hepatitis and acute renal failure. About 5 ml of the whole blood sample was collected in sterile 5 ml serum vials (TARSON). Repeat sampling was done in the same patient with a gap of 7 days from the day of first sampling. Blood samples were transported within 1 h by any one of the researchers and the sera were separated and preserved at −20°C until further use. Of the 181 patient samples in the age group 1-15, 57% were from males and 43% from females. Among the 104 male samples, 25% were in the age group 1-5, 44% in the age group 6-10 and 31% in the age group 11-15. Similarly, for 77 female samples, 16% were in the age group 0-5, 52% in the age group 6-10 and 32% in the age group 11-15 [Table 1].

Antigen preparation

The antigens used for the study namely Leptospira interrogans serovars autumnalis, australis, canicola, javanica and patoc 1 were taken for the study and cultured in EMJH containing 1% Bovine Serum Albumin (BSA) and incubated at 30°C for 10 days. The antigen extract from these strains were obtained as described by Myers (1987) with some modifications. Each strain was grown in 250 ml of medium and harvested by centrifugation at 11,000 × g for 15 min. The pellet was treated with 10 ml Triton X-100 (4%) in 0.01 M phosphate buffered saline (PBS), pH 7.2 and heated in a water bath for 4 h at 50°C, with periodic shaking. This suspension was centrifuged at 11,000 × g for 20 min. The supernatant was preserved with sodium azide (1%). For the purpose of standardisation, antigens were titrated by doubling dilutions in PBS against 10 MAT and Polymerase Chain Reaction (PCR) positive sera and 10 MAT and PCR negative serum samples, for finding the concordant results to pass the quality control. The working concentration for standardisation of antigen for CIE was found to be 1:4 dilutions to get a clear positivity by precipitin arc for the positive samples.

Counterimmuno-electrophoresis

Glass slides (75 by 50 mm) were covered with a thin layer of 0.9% molten agarose (Sigma Chemical Co., St. Louis, USA.) in 0.05 M Veronal buffer (pH 7.5) containing 0.01% sodium azide and allowed to dry. 8 ml volume of the same agar solution was layered onto each slide with a clean pipette. It was allowed to gel at room temperature. Slides were placed in petri dishes and refrigerated at 4°C for at least 10 min. A template was then placed under each glass slide, and wells of diameter 2 mm were made in the gel with a punch. The design consisted of two parallel rows of six wells each separated by a distance of 5 mm. Within each row, the distance between wells was 5 mm.

Electrophoresis was carried out with 0.05 M Veronal buffer (pH 7.5) in the buffer reservoirs. The wells corresponding to the anode were filled with the serum samples diluted in PBS buffer. The pH of the buffer was 7.2 in serial two fold up to 1:32. A constant electric current (6 mA/slide) was applied to each side of the slide for 30 min, at a potential difference of 10 V, with the same buffer. Connecting filter-paper saturated in buffer was placed on both ends of the slide. Subsequently, the cathode side of slide was filled with the antigen, and the run was continued for additional 90 minutes. Positivity of the CIE test consisted of visual detection of one or more precipitin arc between the wells. Results were read immediately after
electrophoresis and again after setting overnight. Positive and negative controls were used in each run.

**Microscopic agglutination test**

MAT was performed on the paired samples using a battery of five live leptospiral strains as antigens. The pathogenic strains were: *L. australis*, *L. autumnalis*, *L. icterohaemorrhagiae* and *L. javanica*. The non-pathogenic (genus specific) strain used was *L. seymourii* (Patco 1). The antigens used were 5-7 days old auto agglutination free cultures grown in EM HH liquid medium (Difco, Sparks, UK) with approximately 1 x 10⁸ - 2 x 10⁸ organisms/mL. The test was performed by a modified Galton (1965) technique. Twenty-five micro litres of sera were diluted with Phosphate Buffer Saline (PBS) pH 7.2 and loaded into six wells of micro titre plate from 1:20 to 1:640, respectively. Twenty-five micro litres of each serogroup antigen was added into each well.

The specimens were mixed gently. After leaving for 2-3 h at room temperature, 3 µl of the suspension was dropped on a slide. The agglutination was observed under dark field microscope at a final magnification of 200×. Sera showing positive reaction were then retested against the respective serogroup using doubling dilutions starting from 1:20 up to end titres. A titre of 1:80 or above was considered as positive.

**Polymerase chain reaction**

PCR (Eppendorf Master Cycler, Germany) was attempted as Gold Standard of diagnosis for the comparative study. CIE positive and MAT negative cases (acute samples of GH 21, 84, 151 and 168) were confirmed using the forward primer 5'-CTG AATCGCTAGATATAAGGT -3' and reverse primer 5'-GG AAACAAAATTGGTGGGAAG-3' (Ab gene, UK) for the amplification of *Leptospira deoxyribonucleic acid (DNA)*. In a sterile 0.5 ml microtube, the following contents were added: 4 µl of 10 x PCR buffer, 7 µl of Template DNA, 1 µl of forward and reverse primer, 2 µl of 25 mm magnesium chloride, 4 µl of deoxynucleotide triphosphates (dNTPs), 2 µl of Taq DNA polymerase and 29 µl of triple distilled deionised water. Vortex the tubes gently and the tubes were spun for 10 seconds to settle the contents and the tubes were placed in a controlled temperature heat block of the thermocycler. The thermal profile involved 30 cycles of each as described by the following steps. Denaturation at 94°C for 1 minute 30 seconds, Primer annealing at 56°C for 1 minute and Polymerisation at 72°C for 1 minute. The final steps were allowed at 72°C for 3 minutes extension to ensure the complete polymerisation. Amplified PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualised under ultraviolet (UV) transilluminator and documented. *L. seymourii* (Patco 1) was used as positive control and distilled water was used as negative control.

**Statistical methods**

The indices of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and efficiency test for CIE and MAT were calculated as follows:

- % Sensitivity = true positive/(true positive + false negative) x 100
- % Specificity = true negative/(false positive + true negative) x 100
- PPV = true positive/all positive test
- NPV = true negative/all negative test
- Efficiency of test = (true positive + true negative)/total samples.

The McNemar test was used to compare the confidence interval and P values of CIE and MAT.

**Results**

When CIE and MAT tests were conducted separately for the sera samples with reference to PCR as the Gold Standard, the following results were obtained. The sensitivity, specificity, PPV, NPV and Efficiency test for CIE were 96.80%, 89.28%, 95.22%, 92.59% and 94.47% and for MAT were 95.90%, 89.83%, 95.08%, 91.37% and 93.92% (Table 2). Four cases from the Government General Hospital (GH 21, 84, 151 and 168) were clearly positive in the first set of samples collected during the acute phase (7-11 days) of the illness when tested by CIE (Table 3). The four samples were found to be clearly positive only in the repeat samples taken after 7 days when tested by MAT. Interestingly, for the acute and convalescent samples as that of CIE. However, no CIE negative samples were found to be MAT positive in the present study (Table 3).

<table>
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<tr>
<th>Table 2: Sensitivity and specificity of CIE and MAT</th>
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<td><strong>Diagnostic techniques</strong></td>
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<td>CIE</td>
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<td>MAT</td>
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<th>Table 3: Acute phase result of CIE and MAT</th>
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<td><strong>Diagnostic techniques</strong></td>
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CIE: Counter immuno-electrolysis, MAT: Microscopic agglutination test. Source: Absolute figures from sample study.
The confidence intervals at 95% and 99% and proportion of positive cases according to McNemars test is presented in Table 4. The four CIE positive and MAT negative cases (GH 21, 84, 151 and 168) acute phase samples were confirmed as positive by PCR test which was taken as gold standard of diagnosis. Among the control group of sera, 213 of the 220 were negative by both tests. The agreement between both the tests was 96.8%.

Discussion

Leptospirosis has been either un-diagnosed or under-diagnosed and under-reported in India. This is due to lack of awareness of the disease, inadequate epidemiological data, and most importantly, non-availability of appropriate standardised laboratory diagnostic facilities in most parts of our country. Although, both MAT and CIE serve the same purpose, MAT is generally considered as WHO reference test and gold standard test worldwide. It is also a traditional technique for diagnosing leptospirosis. The general opinion among researchers is that MAT is the only useful technique for epidemiological studies. But recent studies reveal that even in epidemiological studies, the statistics shown on MAT is not reliable. Human error is possible in MAT because it depends on microscopic visual examination which differs from person to person and from one laboratory to the other laboratory. This problem is absent in CIE. CIE does not require the costly dark field microscope, live hazardous Leptospira and sub culturing on costly medium is not required, standardisation is simple in comparison with MAT and the test system is not only non-cumbersome but also rapid.

McNemars test was also used to find out the significant difference between CIE and MAT in the diagnosis of leptospirosis. At both 95% and 99% confidence intervals, CIE and MAT does not differ significantly. Both the tests are reliable since they express similar confidence levels. The P values calculated had shown 2% increase in CIE than MAT keeping PCR as the reference test. Though, this might not be a significant statistical difference, it cannot be ignored [Table 4]. Four suspected cases were declared positive by CIE and not by MAT at their acute phase of illness. When these sera samples were tested by PCR, they were clearly positive which was considered as the gold standard of diagnosis in the present study.

It was interesting to note that the above mentioned four suspected acute and convalescent samples were clearly positive by PCR as that of the standardised CIE of the current study.

PCR was found useful for early and rapid diagnosis of leptospirosis. PCR detects L. interrogans DNA in body fluids ([Serum and cerebrospinal fluid (CSF)] early in the course of illness with appropriate clinical suspicion. The use of PCR to diagnose leptospirosis may eliminate the need for MAT and culture which are cumbersome and delayed diagnosis.

The performance of a diagnostic test is judged by its sensitivity and specificity. The sensitivity of the MAT (95.90%) for the diagnosis of leptosomal antibodies was lower than that of the sensitivity of the CIE (96.80%). The paired samples collected after 7 days the sensitivity of both the test systems was found to be equal (96.80%) at the later stage of illness. The probability that CIE will be positive given a patient with the leptosomal antibody is higher than that of MAT. But specificity and PPV for both the tests appear to be more or less the same. Importantly, for NPV and – Efficiency Test (ET), the CIE test was found to be not inferior in comparison with MAT. This clearly indicates that the CIE standardised in this study was reliable. Thus, when laboratory diagnostic facilities are too limited for examining sera by the MAT, the CIE could offer a useful method for screening human sera for leptospirosis irrespective of the infecting serogroups.

Earlier studies have identified agreements between the results from both the tests, CIE technique employed a soluble antigen, which was found very useful in screening the human sera for anti-leptosomal antibodies to multiple leptospiiral serogroups. In the present study, the soluble extract prepared from the serovars L. interrogans serovars autumnalis, australis, canicola, javanica and patoc 1. These serovars were used mainly because of the broad cross reactivity against other serovars in the MAT that are frequently associated with human infections. The present study corroborates an earlier such study by Meyer (1987) in comparison of CIE with MAT for suspected leptospirosis in giving 97.9% efficiency.

Our findings are in agreement with an earlier study on swine Leptospirosis which reported that the precipitating antibodies were detected in 5.1 days. Around 7th day, 90% of the sample were positive while by MAT they were still negative. On the 9th day, positivity of CIE reached 100% and only 50% by MAT. Interestingly, from the day 11th to 13th, the CIE and MAT results were equivalent.

Conclusion

The authors concluded that the CIE could be advantageous over MAT due to many reasons. It is safe, rapid, simple, economical and ideal for large number of samples. It can
detect antibodies earlier than MAT and is as reliable as MAT. Since both the tests had shown similar efficacies, only in the later stage of the illness the importance could be given to CIE which was found to be more reliable than MAT in diagnosing positive cases of leptospirosis at an earlier phase of illness. CIE definitely has an additional advantage of carrying out such studies in the field set up also.

References


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