APPENDICES
APPENDIX 1

CHETTINAD HOSPITAL AND RESEARCH INSTITUTE
Kelambakkam, Kanchipuram Dist - 603 103
Institutional Human Ethics Committee

Chair Person
Dr. Kumarasamy
Director V/C TRC & Director V/C National Institute of Epidemiology, Chetpet, Chennai.

Member Secretary
Dr. Ruckmani A
HOD – Pharmacology, CHRI.

Members
Dr. Thayumanavan S
HOD – Paediatrics, CHRI.

Dr. Manohar C
HOD – Forensic Medicine, CHRI.

Dr. Pandiyarn N
HOD – Reproductive Medicine, CHRI.

Dr. Ganesan R
Prof. General Surgery, CHRI.

Dr. Annamalai C
Asst. Prof. Physiology

Dr. Suthakaran C
Head, Clinical Research Madras Diabetic Research Foundation

Dr. Narasimman D
Prof. of Botany
MCC, Tambaram.

Dr. Ramesh N N R
Legal Advisor

Mr. Ramesh
Member from Non-Govt. Vol. Agency

Mr. S.S. Subramanian
Lay person from the Public

Dr. Priyadarshini Shanmugam
Asst. Professor, Microbiology, CHRI


Through “concerned Guide”


Your PhD proposal on the topic “Isolation and molecular characterization of Leptospira from clinical samples employing molecular methods” has been scrutinized by the Institutional Human Ethics Committee and it is pleased to approve your proposal with the following suggestions:

1. To get the informed consent and the information sheet should be in local language.
2. It is suggested to reduce the sample size after discussing with the Institutional Bio-Statistician.

You are also requested to intimate to the Institutional Human Ethics Committee the date of the commencement of your study and to submit the Bi-annual progress.

Dr. A. Ruckmani
Member-Secretary
Institutional Human Ethics Committee
INTERNAL REVIEW BOARD CERTIFICATE ON SYNOPSIS

This is to certify that Dr. Priyadarshini S, Part Time Ph.D candidate (Reg. No. 99090102) presented synopsis of her thesis to IRB on 09.01.2017, entitled "Isolation & Molecular Characterization of leptospirosis from clinical samples". The IRB recommended for the title change as "A comparative evaluation of phenotypic, serological and molecular methods in the diagnosis of leptospirosis". The IRB is satisfied with her synopsis & Presentation.

This is in accordance with the guidelines prescribed in the Ph.D Regulations.

(\text{\textsuperscript{Dr.N.Srinivasan}})
Convener - Research
Chettinad Academy of Research and Education
Kelambakkam.
APPENDIX 3

Sample size estimation

How large a sample would be to find the positive finding within the 25% of the true value with 95% Confidence?

Pilot study shows MAT positivity is 25%.

Required information

<table>
<thead>
<tr>
<th>Anticipated population proportion</th>
<th>P</th>
<th>= 24%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confidence level</td>
<td>100(1-α)%</td>
<td>= 95%</td>
</tr>
<tr>
<td>Acceptable error</td>
<td>L</td>
<td>= 25% of 24</td>
</tr>
</tbody>
</table>

\[n = \frac{Z^2 P (100 - P)}{L^2}\]

\[= \frac{(1.96)^2 \times 24(100-24)}{(6.00)^2}\]

\[= 194 \text{ patients}\]

Taken more number of sample 207 than required 194 patients
APPENDIX 4

CONSENT FORM

INFORMATION FOR PARTICIPANTS OF THE STUDY

☐ Title of the project: A comparative evaluation of microscopic, serological and molecular methods in the diagnosis of Leptospirosis

☐ Name of the investigator: Dr. Priyadarshini Shanmugam

☐ Purpose of this project/study: to determine the most reliable and early diagnostic test for Leptospirosis

☐ Procedure/methods of the study: Microscopy – Dark field microscopy, Fontana’s stain, Serology- IgM ELISA, MAT, MSAT, Molecular method- PCR

☐ Expected duration of the subject participation: 1 year

☐ Any risks expected from the study to the participant: NIL

☐ Maintenance of confidentiality of records: Records will be maintained confidentially

☐ Freedom to withdraw from the study at any time during the study period - Yes

☐ Possible current and future uses of the biological material and of the data to be generated from the research and if the material is likely to be used for secondary purposes or would be shared with others – Will not be shared

☐ Address and telephone number of the investigator: Dr Priyadarshini Shanmugam, Department of Microbiology, Chettinad Hospital and Research Institute, Rajiv Gandhi Salai, Kelambakkam, Kanchipuram 603103. Mobile No: 9841551891
PARTICIPANT CONSENT FORM

Participant’s Name & Address:

☐ Title of the project: A comparative evaluation of microscopic, serological and molecular methods in the diagnosis of Leptospirosis

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the participant: ______________________ Date: _____________
APPENDIX 5

Proforma for Sample Collection

Personal History

Name: Age: Sex:

Address:

Mobile No:

Occupation: Income:

House: Rat menace in house: Yes/ No

Bathed in Ponds/ Swimming Pools:

Treatment already taken:

Clinical history

Fever: Present/ Absent

Duration: Type: Chills: Rigor:

Nausea: Vomiting: Abdominal Pain: Diarrhoea:

Headache: Bodypain: Conjunctival Redness:
APPENDIX 6

Preparation of Fontana’s stain

Stock solutions to be prepared

a. Fixative
   - Acetic acid 1 ml
   - Formalin (40% HCHO) 2 ml
   - Distilled Water 100 ml

b. Mordant
   - Phenol 1 g
   - Tannic Acid 5 g
   - Distilled water 100 ml

c. Ammoniated Silver nitrate
   - 10% Ammonia was added to 0.5% solution of Silver nitrate in distilled water till the precipitate just formed dissolved. More silver nitrate solution was added drop by drop until the precipitate returned and did not re dissolve.
APPENDIX 7

Preparation of Ellinghausen Mc Cullough Johnson and Harris (EMJH) medium

Commercially available medium was used- Difco *Leptospira* Medium Base EMJH

**Composition of Base**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium Phosphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Monopotassium Phosphate</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Ammonium Chloride</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.005g</td>
</tr>
</tbody>
</table>

**EMJH Enrichment**

Difco Enrichment was used which contained Bovine Serum Albumen. The enrichment was added to a final concentration of 10% to the *Leptospira* basal medium.

**EMJH Liquid medium**

Liquid EMJH medium was prepared by dissolving 2.3 g of EMJH base in 900 ml of distilled water. The pH is adjusted to 7.5. After autoclaving at 15 lbs pressure for 15 minutes, enrichment is added to a final concentration of 10%. 100 ml of enrichment was added to 900 ml of EMJH Medium base.
APPENDIX 8

Preparation of Phosphate buffered Saline (for MAT)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>8.0 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.21 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.34 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Dissolve all the ingredients in 100 ml of water and then make up to 1000 ml. This was autoclaved at 121°C at 15 lbs pressure for 15 minutes. The final pH was adjusted to be 7.2 to 7.4.
APPENDIX 9

PREPARATION OF ANTIGEN FOR MICROSCOPIC SLIDE AGGLUTINATION TEST (MSAT)

1. Leptospires were grown in EMJH medium. The required numbers of tubes were initially dispensed with 3 ml of liquid EMJH medium. Each of these tubes was inoculated with a few drops of 5-7 day old culture of *Leptospira* belonging to different serovars. After inoculation the cultures were examined by Dark field microscopy. If they appeared satisfactory, the cultures were dispensed into Erlenmeyer flasks containing 50 ml EMJH medium and were incubated at 30\(^\circ\)C for 5 to 7 days in an incubator. After the period of incubation was over, these cultures were examined, and the purity was checked. This was then dispensed into 500 ml of EMJH medium into a 1 Litre flask and once again incubated at 30\(^\circ\)C for 5 to 7 days in an incubator. These cultures were examined by Dark field microscopy. If found to be satisfactory, formalin was added to a final concentration of 0.5% and the cultures were allowed to stand for 30 minutes.

2. The culture was then centrifuged at 10000 RPM for 30 minutes to pack the Leptospires

3. The supernatant was discarded and the tube was allowed to drain in a slanting position.

4. The sediment in each tube was resuspended in 1.5 ml of a solution containing 0.5% formalin, 12% Oakland 20% Glycerol. Glycerol was added to prevent rapid drying of the antigen, while the slide test was being performed and to provide stability to the antigen. Hypertonic saline was added because it had a surface tension which prevented antigen serum mixtures from spreading too much during rotation of the glass plate

5. All the suspensions were pooled in a 100 ml flask. Glass beads were added and this was shaken vigorously.
6. The antigen is kept at 4°C for 2 weeks before standardization. If a deposit formed after 2 weeks, the supernatant was pipetted into another bottle and the deposit was discarded.

7. The optical density was measured at 520 nm in a spectrophotometer. The reading was adjusted to 0.550 to 0.600 by diluting the suspension with NaCl-formalin-glycerol solution.
APPENDIX 10

Preparation of Antigen for MAT

The stock culture collection of leptospires was maintained in screw-capped test tubes containing 5-6 ml of liquid medium. Fresh sub cultures were made by inoculating 0.5 ml from the each strain / serovar into tubes. At the same time, a loop-full of the culture was examined by dark field microscopy to confirm the presence of viable leptospires and the absence of contamination and to ensure that it was free from "breeding nests". The inoculated cultures were incubated at 30°C and checked for the presence of the growth after 5-7 days. The cultures used as antigens were also checked by MAT against homologous antisera frequently for quality control. The culture was directly used as the antigen. These live antigens were used up to one week after 5-7 days of incubation.
Limitations of the Panbio IgM ELISA kit

- The results from this kit were to be interpreted in association with other clinical data and patient symptoms.
- ELISA results of immunosuppressed patients should be interpreted with caution.
- ELISA was not recommended for use as a screening test in the general population because the positive predictive value (PPV) of the test depended on the likelihood of the organism being present.
- The test should have been performed only on patients with clinical symptoms or when exposure was suspected. There may be a variation in the population seroepidemiology over time in different geographical regions. So the cut-off may require adjustment based on local studies.
- It was advisable not to interpret the results by just visual reading alone.
- In patients with leptospirosis, IgM antibody was the first to appear, in 5 to 10 days after the disease onset and therefore, the detection of IgM was indicative of recent infection.
- A positive result in a single sample should be reported as either suggestive of recent infection or as a presumptive diagnosis.
- Demonstration of rising levels of specific IgM antibody in paired serum samples was regarded as serological evidence of recent infection. When rise or fall in specific IgM antibody could not be demonstrated in consecutively collected serum, a recent Leptospiral infection could be excluded. This test is designed as a screening test; Serum samples collected from a few patients with other acute infections (e.g. Q fever) may give a positive IgM ELISA result.
Reagents / Consumables for IgM ELISA

Primary sample:
- Serum only was used as a test sample

Reagents/ Consumables:
- *Leptospira* Antigen-coated microwells.
- Wash buffer: One bottle of 60 ml of 20x concentrate of phosphate buffered saline with Tween 20 and preservative. One part of wash buffer was diluted with 19 parts of distilled water (Diluted wash buffer thus prepared could be stored for one week at 2-25°C).
- Sample diluent: Tris buffered saline
- Horseradish peroxidase (HRP) conjugated Anti-human IgM
- TMB Chromogen
- Reactive (Positive) control
- Calibrator
- Negative control
- Stop solution: 1M Phosphoric Acid
- Distilled water

Equipment:
- ELISA reader with 450 nm filter.
- Automatic microplate washer
- Micropipettes
- Disposable pipette tips.
- Timer
- Flask
- Graduated cylinder
- Test tubes or microtitre plate for making the dilutions of serum

Storage conditions:
- The kit should be stored at 2-8°C and should not be kept in the freezer
- Immediately after use, the reagents must be returned to 2-8°C
APPENDIX 12
DNA Extraction for PCR

DNA Extraction by Nucleospin Column Method (MACHEREY-NAGEL GmbH & Co)

NucleoSpin® Kits provide a fast, efficient, and economical means of purifying nucleic acids from small volumes of bacterial or yeast cultures, PCR products, agarose gels, blood, tissues, viruses, or plants.

Each spin column contains a specially activated silica membrane filter that traps nucleic acids and separates them from contaminating proteins and other cellular debris. After contaminants are spun through the filter, nucleic acids are eluted in a low-salt buffer (DNA) or RNase-free water (RNA).

With the Nucleo spin Blood method, genomic DNA is prepared from whole blood, cultured cells, serum, plasma, or other body fluids. Lysis is achieved by incubation of whole blood in a solution containing large amounts of chaotropic ions in the presence of Proteinase K. Appropriate conditions for binding of DNA to the silica membrane of the corresponding Nucleo Spin Blood columns are achieved by addition of ethanol to the lysate.

The binding process is reversible and specific to nucleic acids. Washing steps efficiently remove contaminations. Pure DNA is finally eluted under low ionic strength conditions in a slightly alkaline elution buffer.
Protocol (Manufacturer’s Protocol)

Fresh blood/ serum/ plasma/ other body fluids.

200 microlitre blood + 25 microlitre Proteinase K+ 200 microlitre B3 buffer

Mix and water bath at 70°C for 10-15 minutes.

210 micro liter of Ethanol

Transfer all the solution to the Nucleospin columns and run at 11,000 rpm FOR 1 min.

Discard the flow through and keep new collection tube to the spin column

Add 500 microlitre of Buffer BW 1st wash and run at 11,000 rpm for 2 minutes.

Place the nucleospin column into a new collection tube and add 600 microlitre of Buffer B5 and run at 11,000 rpm for 2 minutes.

Discard the flow through and reuse the column and run at 11,000 rpm for 2 minutes for excess removal of ethanol.

Place the column into the new 1.5 ml micro centrifuge collection tube and add 100 microlitre of Preheated Buffer BE and run at 11000 rpm for 2 minutes.

Store the genomic DNA @ -20° for Molecular analysis.

With the successful genomic DNA extraction from commercial kit, DNA samples were critically analysed for its purity and concentration by using UV Spectrophotometer.
UV spectroscopy is a type of absorption spectroscopy in which light of ultraviolet region (200-400 nm.) is absorbed by the molecule. Absorption of the ultraviolet radiations results in the excitation of the electrons from the ground state to higher energy state. The energy of the ultra-violet radiation that are absorbed is equal to the energy difference between the ground state and higher energy states (\(\Delta E = hf\)).

UV spectroscopy obeys the Beer-Lambert law, which states that: “when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution.”

Purity of the DNA was checked using UV Spectrophotometer.

- To quantify the amount of DNA the reading was taken at a wavelength of 260 nm and 280 nm.
- 10 µL of DNA was diluted in 90 µL of distilled water (The dilution factor was 10).
- Pure preparations of DNA and RNA have values of 1.8 and 2.0 respectively.
- Concentration of DNA was calculated by using the formula.
- To quantify the amount of DNA & RNA, readings are taken at wavelength of 260 nm & 280 nm.

\[
\text{OD of 1 corresponds to } \sim 50 \mu g/ml \text{ for ds DNA.}
\]

\[
\sim 40 \mu g/ml \text{ for ss DNA & RNA.}
\]

\[
\sim 30 \mu g/ml \text{ for Oligonucleotides.}
\]
The ratio between the readings at 260 & 280 nm provides an estimate of the purity of Nucleic acid (260/280nm).

DNA concentration measured by:

\[ \text{Conc (µg/ml)} = (\text{A260 reading} \times 50 \times \text{dilution factor}) \]

Once the purity of the DNA was checked, PCR was carried out. While designing the primers following guidelines were considered and based on Review of Literature and its accuracy was seen using NCBI- BLAST.
APPENDIX 14

Procedure for Gel Electrophoresis

Gel electrophoresis is a method that separates macromolecules on the basis of size, electric charge and other physical properties; it refers to a technique in which molecules are forced across a span of gel, motivated by an electrical current. Many important biological macromolecules e.g. amino acids, peptides, proteins, nucleotides and nucleic acids possess ionisable groups and, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-).

The driving force for electrophoresis is the voltage applied to electrodes at either end of the gel, depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode, when an electric field is applied across a gel at neutral pH, the negatively charged phosphate groups of the DNA cause it to migrate toward the anode. The location of DNA within the gel can be determined by staining with a low concentration of ethidium bromide, a fluorescent intercalating dye and read at UV transilluminator.

Procedure:

➢ Preparation of 10X TAE Buffer: Table no:2

<table>
<thead>
<tr>
<th>SN</th>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris base(mol wt 121.12)</td>
<td>24.2 g</td>
</tr>
<tr>
<td>2</td>
<td>Glacial acetic acid</td>
<td>5.71 ml</td>
</tr>
<tr>
<td>3</td>
<td>EDTA disodium salt</td>
<td>3.72g</td>
</tr>
<tr>
<td>4</td>
<td>Distilled Water</td>
<td>80ml</td>
</tr>
<tr>
<td>---</td>
<td>----------------</td>
<td>------</td>
</tr>
<tr>
<td>Make up to 1000 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Preparations of ethidium bromide (stock solution)**

  Weigh 10 mg ethidium bromide into a sterile tube and dissolve in 10 ml sterile distilled water. The stock is stored at 4C.

- **Preparation of sample loading dye Glycerol & bromophenol blue (6x)**

  3 ml glycerol (30%), 25 mg bromophenol blue (0.25%) dH2O to 10 mL

- **Preparation of agarose solution for casting the gel:**

  - Seal the edges of a clean, dry plastic gel-tray either with tape or other means.
  - Position the appropriate comb so that complete wells are formed when the agarose solution is added
  - Dilute 10x TAE buffer to prepare the appropriate amount of 0.5x TBE buffer to fill the electrophoresis tank and to prepare the gel
  - Weigh powdered agarose (1.5%) and add it to an appropriate amount of 0.5x TAE buffer in an Erlenmeyer flask with a loose-fitting cap.
  - Heat the slurry in a microwave oven or in a boiling water bath until the agarose dissolve.
- Cool the mixture to 50 - 60°C and add ethidium bromide (from a stock solution of 10 mg/ml) to a final concentration of 0.2 µg/ml and mix thoroughly.

- Pour the solution into the gel-tray and allow the gel to set. The amount of gel used should correspond to a depth of approximately 3 - 5 mm.

- After the gel is completely set, carefully remove the comb and the tape and place the gel in the electrophoresis tank.

- Add enough 0.5x TAE buffer to the electrophoresis unit to cover the gel to a depth of about 2 - 5 mm.

- PCR amplicons along with pre loaded dye were loaded into the wells and run at 50 volts for 45 minutes.
APPENDIX 15

Unusual presentation of Leptospirosis

PRIYADARSHINI SHANMUGAM, PARTHASARATHY M.P., JEYA M.

ABSTRACT

Leptospirosis is the most common zoonotic disease. Leptospirosis has been ignored as a cause of acute renal failure. We report a case of leptospirosis with acute renal failure in an female state. The patient presented with severe abdominal pain, nausea and reduced urine output. Serum urea and creatinine are elevated. IgM antibodies for Leptospira are positive by ELISA test. The patient was treated with augmentin 1.2 gram IV BD for ten days. The patient restored the renal functions gradually and abdominal pains subsided within 3 days after starting the augmentin therapy. We report this case for the unusual presentation of leptospirosis with acute renal failure in an female state.

INTRODUCTION

Leptospirosis is a febrile zoonosis of worldwide distribution. Traditionally considered an occupational risk among persons exposed to contaminated water or infected animal urine, it is becoming recognized as a major public health problem in the tropics.

Clinical recognition of leptospirosis is difficult, due to its multi-system involvement. It presents with varied clinical manifestations and is often misdiagnosed as influenza, septic meningitis, encephalitis, dengue fever, hepatitis or gastroenteritis. Also epidemic associated with high case fatality (> 15%) break out annually during the season of heavy rainfall in areas that lack basic sanitation infrastructure.

Here we are presenting a rare manifestation of leptospirosis with acute renal failure without fever, with severe abdominal pain.

CASE REPORT

A 48 year old man presented with complaints of severe abdominal pain, nausea and reduced urine output. No history of vomiting, haematuria, no melaena; pain increased after food intake.

On general examination patient is afibrile, conscious and dehydrated. Pulse rate and blood pressure are within normal limits. On local examination of abdomen, diffuse tenderness more in the upper abdomen is present. No guarding or rigidity. Bowel sounds are heard.

The following investigations are carried out. Hb-11.000 gms/dl, WBC 10,000 mm3, BUN-122 gms %, Blood urea-77 mg/dl, serum creatinine-1.14 mg/dl. Liver function tests are within normal limits. Kidney biopsy showed features suggestive of mild infection, mild pyelonephritis. USG abdomen/pelvis revealed bilateral marginal renomegaly with features suggestive of acute renal failure.

Patient underwent hemodialysis twice during his hospital stay and his blood urea, serum creatinine levels reduced gradually to normal limits. Patient was still having abdominal pain. Serological investigation for leptospirosis was done. IgM antibodies are positive by ELISA method.

Patient was started on augmentin 1.2 gram IV RI along with IV fluids for 10 days. After starting augmentin the abdominal pain was relieved and the renal function tests returned to normal even after stopping hemodialysis. Patient was discharged.

This case is presented for its rare and unusual presentation of leptospirosis as afibrile condition with acute renal failure with complaints of abdominal pain. This gives a clue to the clinician to suspect leptospirosis in rare presentation like this.

DISCUSSION

Leptospirosis clinically presents as a biphasic illness. The first phase of the disease is commonly referred to as the septicemic phase. It is characterized by fever, headache, myalgia, conjunctival suffusion and nonspecific features that may include mild cough, lymphadenopathy, rash, anorexia, nausea and vomiting. This phase is followed by a brief febrile period of variable duration that is, in turn, is followed by the immune phase of the illness. The common organ involved during this phase are liver and kidneys. Both organ derangements are reversible.

This patient presented with severe abdominal pain and oliguria in an afibrile state. No history of fever even before getting admitted to our hospital. The patient gave a history of water stagnation near his house as well as in his work place. The afibrile state of the patient may be due to repeated exposure of the patient to Leptospira in small number.

Severe leptospirosis may carry high mortality if treatment is not initiated early. It is imperative that high index of suspicion for the disease be maintained particularly in endemic areas, there is a need for increasing the awareness of the disease so that the timely therapy can be instituted to the patients.

REFERENCES


THE ANTISEPTIC

April 2010

Dr. Priyadarshini Shanmugam, M.D.
Dr. M.P. Parthasarathy, M.B.B.S.
Dr. M. Jeeya, M.D.
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Specially Contributed to "The Antiseptic" Vol. 107 No. 4 & 5: 166 - 166