Abstract: Isolation attempts were carried out between November 2006 and May 2010. Samples collected from clinically suspected cases of cattle (26), sheep (42), dogs (15), pigs (15), humans (53), stagnated water (10) in Rice fields and from rats (299) were inoculated in to EMJH liquid medium with tween- 80, antibiotics and 5- fluorouracil. A total of 17 isolates were recovered, from sheep (5), pigs (4), humans (2), rice field water (1) and from rats (5). The isolates were purified and maintained in EMJH liquid medium and as well as semi solid medium. The Physio-chemical characterization of isolates at 13°C, growth in the presence of 8 - Azaguanine, Lipase activity and animal inoculation studies revealed the pathogenicity of the isolates. The isolates were identified using Dark field microscopy, Fontana’s staining, characteristic Dingers phenomenon and by histopathological studies.

Keywords: Leptospira, EMJH medium, Dark field microscopy, Fontana’s staining, Physico chemical characterisation and Histopathology

1. Introduction

Leptospirosis is a worldwide zoonosis and is considered as a re-emerging disease. In spite of economic losses to the animal production by the bacterium, its zoonotic character makes it an important public health problem [1]. Leptospirosis can be diagnosed by several laboratory methods of which the serological methods are the most widely used [9], [7] and [1] but the isolation of Leptospira allows the definite diagnosis of individual infections. It also helps to study molecular epidemiology of the disease and to develop suitable vaccine to control the disease. In Andhra Pradesh, so far no data related to isolation of leptospires from naturally infected animals and from reservoir hosts like rodents is available.

The information available on Leptospirosis is limited to serology. Hence, attempt was made for isolation of leptospires from infected animals, humans and rats to find out epidemiological link between animals, humans and rats. The pathogenicity of the isolates were confirmed by using physico-chemical characterization, biological and histopathological methods.

2. Materials and Methods

2.1 Collection of Samples

Samples were collected during the period from February 2006 to May 2010. A total of 159 samples from clinically suspected cases of Cattle (26), Sheep (42), Dogs (13), Pigs (15), Humans (53), stagnated water in Rice fields of outbreak area (10). A 10% homogenous suspension of kidneys and livers collected from 299 Rats trapped from different geographical areas.

2.2 Preparation of EMJH Medium

EMJH liquid medium with Tween 80, Antibiotics and 5-fluorouracil was used as a selective medium according to [4] with slight modifications.

2.3 Preparation of Leptospira Base

1.15g of EMJH base (Difco) dissolved in 500ml of triple distilled water (Sterile) was used for preparation of media.

2.4 Preparation of Nutrient supplement consisting of

- BSA fraction V 5.0 g
- CaCl₂ (stock solution) 0.5 ml
- ZnSO₄ (Stock solution) 0.5 ml
- MnSO₄ (Stock solution) 50.0 µl
- FeSO₄ (Stock solution) 5.0 ml
- MgCl₂ (Stock solution) 0.5 ml
- Vit-B₁₂ (Stock solution) 0.5 ml
- Tween 80 (Stock solution) 0.5 ml
- CuSO₄ (stock solution) 0.2 ml
- Nalidixic acid (stock solution) 1.0 ml
- Lactalbumin hydrolysate 0.5 g
- Sodium pyruvate 0.02 g
- L-Cystine 10 mg
- Distilled water 22.5 ml

The base and supplement were sterilized by 0.45 and 0.22 µm pore size filter membrane and mixed at the ratio of 9:1. The medium is dispensed in aliquots in 5ml, 10ml screw capped sterile test tubes. Each of two test tubes, one kept at room temperature and another at 37°C for 48 hrs and remaining tubes at 4°C to check for contaminants. After 48 hrs, if there is no contamination, all the tubes were kept at 25°C-30°C in B.O.D incubator for future use.

2.5 Isolation of Leptospira

All the clinical samples collected were processed and inoculated into EMJH liquid media and incubated at 29°C ± 1°C in B.O.D. incubator and screened for presence of Leptospira at weekly intervals under dark-field microscope.

2.6 Purification of Leptospiral isolates

2.6.1 Filtration: Leptospiral isolates obtained from different sources were purified by filtration through Cellulose...
membrane filters with 0.22µm. (M/S Sartorius, India) and cultured freshly into EMJH media.

2.6.2 Animal inoculation: Pathogenic studies were carried out in Guinea Pigs weighing 150 to 250 gms. (IP). 12 hours of post inoculation 0.5 ml. of heart blood was inoculated into EMJH media for re-isolation.

2.7 Identification of Leptospira Isolates

2.7.1 Dark field microscopy: Isolates obtained were initially observed under Dark-field microscope for their characteristic motility.

2.7.2 Fontana’s staining: done with ADMAS staining kit.

2.7.3 Sub surface growth in semi-solid EMJH medium: for specific growth for cultural character.

2.7.4 Histopathological examination: Liver and Kidney tissues from inoculated Guinea Pigs were collected in 10% formalin for Histopathological studies and stained with Haematoxylin & Eosin for examination.

2.7.5 Physico-Chemical characterization:

2.7.5.1 Growth at 13°C: Along with known pathogenic and saprophytic strains of leptospira in duplicates incubating once at 13°C and another set at 30°C. Growth was checked twice in a week up to 21 days.

2.7.5.2 Growth in the presence of 8-Azaguanine: Incubated the tubes both test and control at 30°C with and without 8-Azaguanine. Growth was checked twice in a week up to 21 days.

2.7.6 Reaction on egg yolk agar media: To study the lipase activity of Leptopiral isolates.

3. Results

3.1 Isolation of Leptospira

A total of 17 Leptospiral isolates, 5 from Sheep, 4 from Pigs, 2 from Humans, 1 from Rice field water and 5 from Rodents were recovered from 458 clinical samples collected from different species with percentage positivity of 3.7% up on culturing. (Table: 1).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Source of isolation</th>
<th>No. of samples subjected for isolation</th>
<th>No. of samples found positive</th>
<th>Percent positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rats</td>
<td>299</td>
<td>5</td>
<td>1.67</td>
</tr>
<tr>
<td>2</td>
<td>Sheep</td>
<td>42</td>
<td>5</td>
<td>11.91</td>
</tr>
<tr>
<td>3</td>
<td>Pigs</td>
<td>15</td>
<td>4</td>
<td>26.6</td>
</tr>
<tr>
<td>4</td>
<td>Humans</td>
<td>53</td>
<td>2</td>
<td>3.77</td>
</tr>
<tr>
<td>5</td>
<td>Rice field</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Cattle</td>
<td>26</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Dogs</td>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>458</td>
<td>17</td>
<td>3.71</td>
</tr>
</tbody>
</table>

The leptospira isolates recovered during the period i.e., five from rats, five from sheep, four from pigs, two from humans and one from rice field water, were further subjected to membrane filtration using 0.22µm membrane filters for purification. Later, sub culturing was done on EMJH medium regularly and finally maintained on EMJH semisolid medium for further studies.

3.2 Animal inoculation

All the 17 isolates recovered on cultural isolation were inoculated into guinea pigs individually for further purification. The blood samples of respective guinea pigs were further inoculated into fresh EMJH liquid medium and re-isolated the organisms in pure form.

3.3 Identification of leptospira

The leptospiral isolates obtained during the isolation were maintained in EMJH liquid medium as well as semi solid medium. In the semi solid medium the characteristic sub surface growth, dinger’s phenomenon was observed (Fig. 1).

The isolates obtained from different species were identified initially by using dark field microscopy and by staining methods.

3.4 Dark field microscopy

The leptospiral isolates obtained were observed under dark field microscopy. The organisms were actively motile. The characteristic motility of flexion, extension and rotational movements were observed under dark field microscopy suggestive of leptospira.
3.5 Fontana’s Staining

The 17 isolates of leptospira recovered in pure form were stained using ADMAS staining kit, and observed the characteristic morphology of spiral shape with hooked ends. All the organisms showed characteristic morphological features of the leptospira and seen as brownish black with yellow color background (Fig. 2).

Figure 2: Fontana’s staining of leptospira isolate

3.6 Histopathology

The histopathological studies of liver and kidney collected from infected guinea pigs revealed focal areas of congestion, severe degeneration with vacuolation in hepatic cell, dilatation of sinusoidal spaces, sinusoidal haemorrhages, infiltration of neutrophils, eosinophils, proliferation of bile ducts and perivascular mononuclear cell infiltration. In majority of the cases binucleated hepatic cells were prominent (Fig. 4.a,b,c,d and e).

Figure 4 (b): Severe degenerative changes in hepatic cells with vacuolation H & E X 280

Figure 4 (c): Dilatation of sinusoidal space with haemorrhages and infiltration of neutrophils and eosinophils H & E X 280

Figure 4 (d): Perivascular, mononuclear aggregation H & E X 280

Figure 4 (e): Several binucleated hepatic cells H & E x 280

In kidneys, focal areas of congestion, glomerular congestion, intertubular haemorrhages, periglomerular infiltration of neutrophils and monocytes, inter tubules infiltration of mononuclear cells i.e. interstitial nephritis, vacuolation and inter tubular fibrosis were recorded (Fig. 5.a,b,c and d).

4.a. Severe congestion H & E X 70

Figure 4: Histopathology - liver of Guinea pigs infected with leptospira
3.7 Physico chemical characterization

The leptospira isolates isolated form different species were subjected for physicochemical characterization, growth at 13°C, 8-Azaguanine test and reaction on Egg-Yolk agar to differentiate pathogenic and non pathogenic leptospires.

3.8 Growth at 13°C

All the 17 leptospira isolates fail to grow at 13°C indicating the pathogenicity. Results of the study shown in (Table 2).

Table 2: Growth characterization of leptospiral isolates

<table>
<thead>
<tr>
<th>S.No</th>
<th>Source of leptospiral isolates</th>
<th>No. of samples tested</th>
<th>No. of leptospiral isolates obtained</th>
<th>Growth at Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13°C</td>
</tr>
<tr>
<td>1</td>
<td>Rats</td>
<td>299</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Sheep</td>
<td>42</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Pigs</td>
<td>15</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Humans</td>
<td>53</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Rice field</td>
<td>10</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Cattle</td>
<td>26</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.9 8-Azaguanine test

The 17 leptospiral isolates were not grown in the presence of 8-azaguanine. The control pathogenic strains L.interrogans also fail to grown in the presence of 8-azaguanine. However, growth of non pathogenic strain L.patoc was noticed (Table 3).
Reaction on Egg – Yolk agar

All the 17 isolates showed lipase activity on egg yolk agar plates indicating pathogenicity (Fig.3).

4. Discussion

EMJH liquid medium with tween 80, antibiotics and 5-fluorouracil was used as selective media for isolation and maintenance of leptospiros. Similarly, EMJH liquid media with tween 80, antibodies was also used by the earlier workers [19], [18], [17] and [10] for isolation and maintenance of leptospires in their studies. [16] reported the harmful effect of antibiotics on leptospiral growth after 48 hrs incubation. However, such effect of antibiotics on growth of leptospiros was not noticed during our investigation.

A total of 299 kidney samples from rats of different places were cultured in EMJH liquid medium. Leptospiros was isolated from five of the 299 samples cultured from rats and five from the 42 samples cultured from sheep, four from the 15 samples cultured from pigs. Similarly, two leptospiros isolates were obtained from 53 blood samples cultured from humans. Out of ten water samples cultured, leptospiros was isolated from one sample.

During the study, leptospiros organisms were observed in EMJH media between 12-14 weeks of inoculation. [11] and [3] also observed leptospiros between 12 to 14 weeks. Keeping in views of slow growth of leptospiros, [13] suggested observing for the period of 4 to six months for isolation of leptospiros.

Leptospiros isolations could not be made from the samples collected during the period of study from cattle (26) and dogs (13). There is a need to improve the methodology suitably. Isolation of leptospiros from clinical samples is an art, requires skill and experience. Successful isolation depends on the time of collection, interval between collection and processing, number of leptospiros present in the samples, the interference of contaminating organisms and tissue autolysis. [8], [12] and [5]. The organisms growing in the EMJH liquid media were initially tested under darkfield microscopy and noticed the flexion, extension and rotational motility characteristic of leptospiros.

In the present study pathogenic studies were conducted in guinea pigs and leptospirosal organisms were re-isolated. Histopathological examination of liver and kidney collected from infected guinea pigs revealed congestion, degeneration, vacuolation, sinusoidal haemorrhages, perivascular mononuclear infiltration in hepatic cells, bimucleated and majority cases hepatic cells were bimucleated. This type of changes in the liver was reported by [14]. Histopathological examination of kidney samples revealed the focal areas of congestion, glomerular congestion, periglomerular infiltration of neutrophils and monocytes, interstitial nephritis, intertubular fibrosis were observed during the study. Similar type of lesions in kidneys were also recorded by [14].

A total of 17 isolates received from sheep (5), pigs (4), rats (5), humans (2) and rice field (1) were maintained in the laboratory for further study. The isolates when stained with ADMAS staining kit showed the spiral shape with hooked ends the characteristic morphological features of the leptospiros and were seen as brownish black organisms with yellow back ground Fig. 12. [2] also used ADMAS staining kit and reported the presence of leptospiros.

Characterization of leptospiros isolations is an important tool for identification of leptospiros strain. Leptospiros isolates that were confirmed conventionally were subjected to physico-chemical characterization an was assessed by studying the leptospirosal growth at 13°C, in the presence of 8-azaguanine and reaction on egg-yolk agar to differentiate pathogenic from non pathogenic leptospiros. During the study all the 17 leptospiros isolates fail to grow at 13°C and in the presence of 8-azaguanine. All the isolates showed lipase activity on egg-yolk agar indicating the pathogenic nature of leptospiros [4], [15], [10] and [6] also differentiated pathogenic leptospiros from non pathogenic by

![Figure 3: Lipase activity of leptospira on egg yolk agar](image-url)

**Test:** Leptospira isolate (S3)

**Negative Control:** Negative Control (Non pathogenic)

### Table 3: Growth characterization of Leptospiral isolates in the presence of 8- Azaguanine

<table>
<thead>
<tr>
<th>S. No</th>
<th>Source of leptospiral isolates</th>
<th>No.of samples Tested</th>
<th>No.of leptospiral Isolates obtained</th>
<th>With 8-azaguanine</th>
<th>Without 8-azaguanine</th>
<th>Ref pathogen With</th>
<th>Ref pathogen Without</th>
<th>Ref. non pathogen With</th>
<th>Ref. non pathogen Without</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rats</td>
<td>299</td>
<td>5</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>2</td>
<td>Sheep</td>
<td>42</td>
<td>4</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>3</td>
<td>Pigs</td>
<td>15</td>
<td>4</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>4</td>
<td>Humans</td>
<td>53</td>
<td>2</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>5</td>
<td>Rice fields</td>
<td>10</td>
<td>1</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>6</td>
<td>Cattle</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>445</td>
<td>17</td>
<td></td>
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<td></td>
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growing at 13°C, studying the effect of 8-azaguanine and lipase activity.

5. Acknowledgement

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References


Author Profile

Dr. D. Rani Prameela received Bachelor degree of Veterinary and Animal Sciences in 1990, Masters in Veterinary Microbiologyin 1992 and Doctoral degree programme in 2010 at College of Veterinary Sciences, Sri Venkateswara Veterinary University, Tirupati and worked as Assistant Professor from 2001 to 2012 and Associate Professor from 2012 to till date. Presently working as Head at State Level Animal Disease Diagnostic Laboratory, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, Ind