

Isolation Studies of Leptospira in Andhra Pradesh

Dr. D. Rani Prameela¹, Dr. D. Sreenivasulu², Dr. P. Vijayachari³, Dr. Ch. Srilatha⁴, Dr. S. Vijaya Lakshmi⁵

^{1,2,5} Department of Veterinary Microbiology, C.V.S.C., Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

³ Director, ICMR, Port Blair, Center for Diagnosis and typing of leptospira, Andaman and Nicobar Islands

⁴ Department of Veterinary Pathology, C.V.S.C., Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Abstract: Isolation attempts were carried out between November 2006 and May 2010. Samples collected from clinically suspected cases of cattle (26), sheep (42), dogs (13), 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 world wide 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 leptospire 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 leptospire 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
CaCl ₂ (stock solution)	0.5ml
ZnSo ₄ (Stock solution)	0.5 ml
MnSo ₄ (Stock solution)	50.0 ml
FeSo ₄ (Stock solution)	5.0 ml
MgCl ₂ (Stock solution)	0.5 ml
Vit-B ₁₂ (Stock solution)	0.5 ml
Tween 80 (Stock solution)	0.5 ml
CuSo ₄ (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 BOD 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. (I/P). 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 13⁰C: Along with known pathogenic and saprophytic strains of *leptospira* in duplicates incubating once at 13⁰C and another set at 30⁰C. 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 30⁰C 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 Leptospiral 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 1: Details of clinical samples collected and *Leptospiral* isolates recovered Purification of leptospira isolates

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

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).

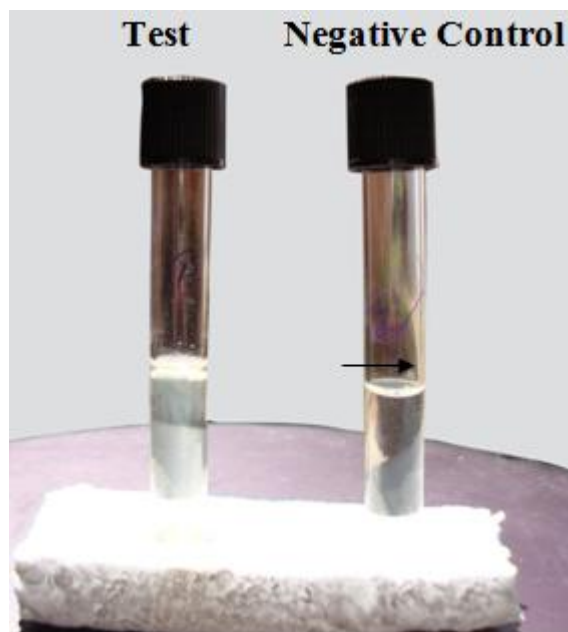


Figure 1: Growth pattern of *Leptospira* isolate S3 in semisolid agar medium – Dinger’s Phenomenon

Test : *Leptospira* isolate (S3)
Negative Control: Negative Control

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 microscope suggestive of leptospire.

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 back ground (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 multinucleated hepatic cells were prominent (Fig.4.a,b,c,d and e).

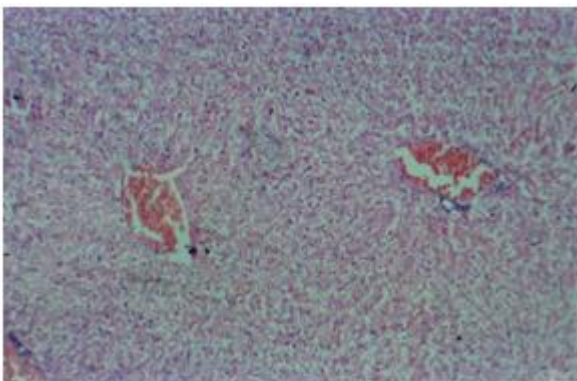


Figure 4: Histopathology - liver of Guinea pigs infected with leptospira

4.a. Severe congestion H & E X 70

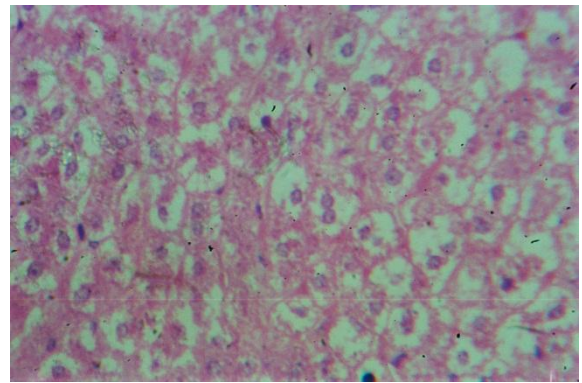


Figure 4 (b): Severe degenerative changes in hepatic cells with vacuolation H & E X 280

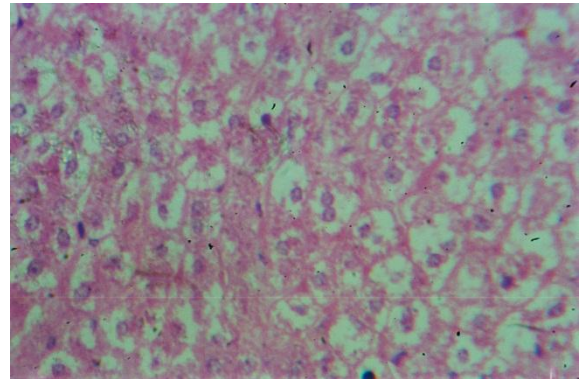


Figure 4 (c): Dilatation of sinusoidal space with haemorrhages and infiltration of neutrophils and eosinophils H & E X 280

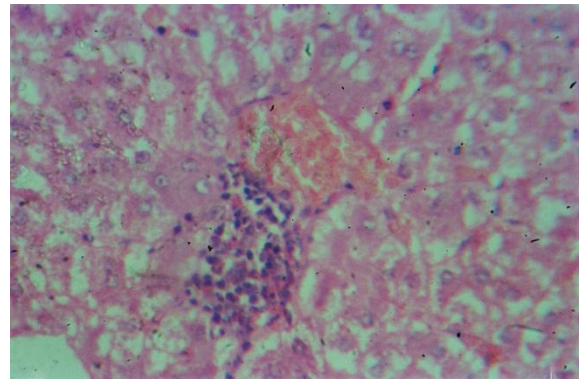


Figure 4 (d): Perivascular, mononuclear aggregation H & E X 280

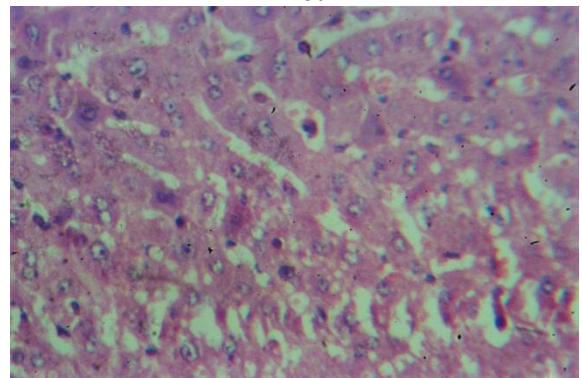


Figure 4 (e): Several binucleated hepatic cells H & E x 280

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).

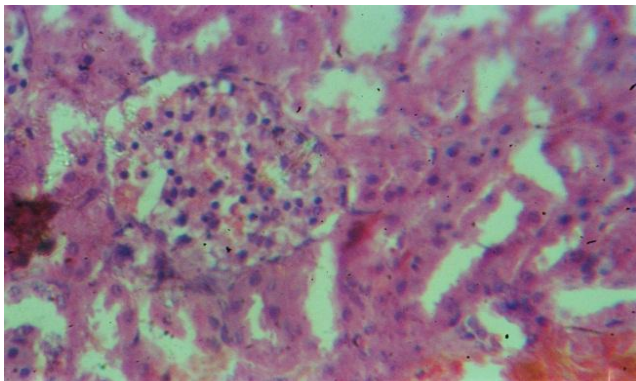


Figure 5: Histopathology – kidney of guinea pig infected with leptospira
 5 (a): Glomerular infiltration H & E X 280

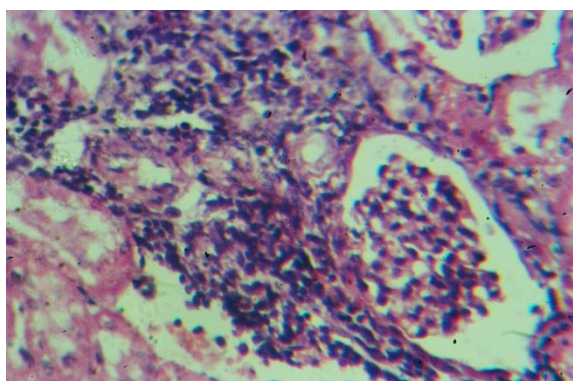


Figure 5 (b): Periglomerular and intertubular infiltration H & E X 280

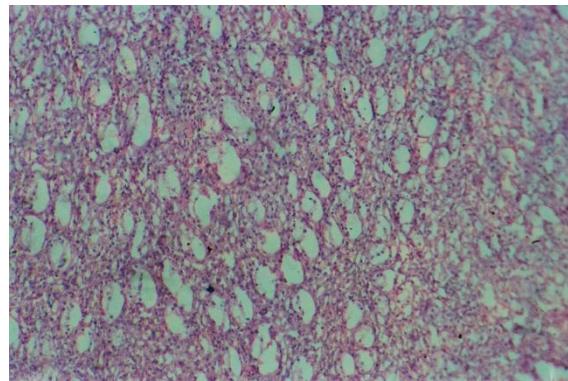


Figure 5 (c): Cystic dilatation of tubules & E X 70

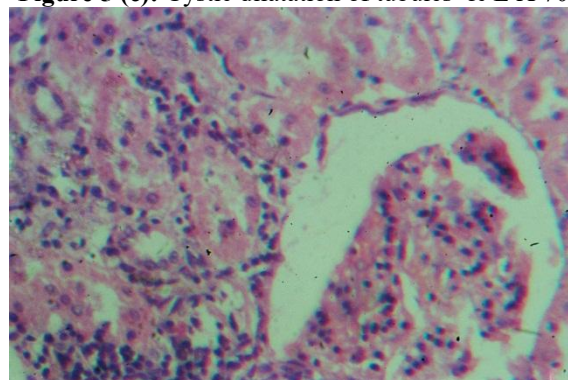


Figure 5 (d): Intratubular infiltration of mononuclear cells H & E X 280

3.7 Physico chemical characterization

The leptospira isolates isolated from 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 leptospire.

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

S.No	Source of leptospiral isolates	No. of samples tested	No. of leptospiral Isolates obtained	Growth at		Controls			
				13°C	30°C	Reference pathogenic		Reference Non -pathogenic	
						13°C	30°C	13°C	30°C
1	Rats	299	5	-	+	-	+	+	-
2	Sheep	42	5	-	+	-	+	+	-
3	Pigs	15	4	-	+	-	+	+	-
4	Humans	53	2	-	+	-	+	+	-
5	Rice field	10	1	-	+	-	+	+	-
6	Cattle	26	-	-	-	-	+	+	-

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).

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

S. No	Source of leptospiral isolates	No.of samples Tested	No.of leptospiral Isolates obtained	Growth		Control			
				With 8-azaguanine	Without 8-azaguanine	Ref pathogen		Ref. non pathogen	
						With	Without	With	without
1	Rats	299	5	-ve	+ve	-ve	+ve	+ve	+ve
2	Sheep	42	5	-ve	+ve	-ve	+ve	+ve	+ve
3	Pigs	15	4	-ve	+ve	-ve	+ve	+ve	+ve
4	Humans	53	2	-ve	+ve	-ve	+ve	+ve	+ve
5	Rice fields	10	1	-ve	+ve	-ve	+ve	+ve	+ve
6	Cattle	26	-	-	-	-	-	-	-
Total		445	17						

Reaction on Egg – Yolk agar

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



Figure 3: Lipase activity of leptospira on egg yolk agar
Test: Leptospira isolate (S3)
Negative Control: Negative Control (Non pathogenic)

4. Discussion

EMJH liquid medium with tween 80, antibiotics and 5-fluorouracil was used as selective media for isolation and maintenance of leptospira. 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 leptospira 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 leptospira was not noticed during our investigation.

A total of 299 kidney samples from rats of different places were cultured in EMJH liquid medium. Leptospira 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 leptospiral isolates were obtained from 53 blood samples cultured from humans. Out of ten water samples cultured, leptospira was isolated from one sample.

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

Leptospiral 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 leptospira 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 leptospira 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 leptospira.

In the present study pathogenic studies were conducted in guinea pigs and leptospiral 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, binucleated and majority cases hepatic cells were binucleated. 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 was 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 leptospira and were seen as brownish black organisms with yellow back ground Fig. 12. [2] also used ADMAS staining kit and reported the presence of leptospira.

Characterization of leptospiral isolates is an important tool for identification of leptospiral strains. Leptospiral isolates that were confirmed conventionally were subjected to physico-chemical characterization and was assessed by studying the leptospiral growth at 13°C, in the presence of 8-azaguanine and reaction on egg-yolk agar to differentiate pathogenic from non pathogenic leptospira. During the study all the 17 leptospiral 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 leptospira [4], [15], [10] and [6] also differentiated pathogenic leptospira from non pathogenic by

growing at 13°C, studying the effect of 8-azaguanine and lipase activity.

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Author Profile

Dr. D. Rani Prameela received Bachelor degree of Veterinary and Animal Sciences in 1990, Masters in Veterinary Microbiology in 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