A REPORT ON OCCURRENCE OF LEPTONEMA IN ANDHRA PRADESH

Presented by

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INTRODUCTION

- **Leptospirosis is a wide spread zoonotic disease**
- **Affecting humans and wide variety of animals.**
- **Important public health problem in developing countries including India.**
- **In India disease has been reported from various states.**

- **Madhya Pradesh, Uttar Pradesh, Punjab, Maharashtra, Bihar, Orissa, West Bengal, Jammu & Kashmir and Delhi.**

- **Tamilnadu, Karnataka, Kerala, Andaman and Nicobar Islands.**

- **Earliest report of Leptospirosis in 1931 by Taylor in Andaman and Nicobar islands followed by Madras city in 1932 by Ayyar.**

- **Leptonema ilini strain 3055 originally identified as leptospira by Hanson in 1965 from the urine of bull.**

- **Infected rodents, domestic wild and animals become inapparent carriers and excrete leptospires in urine.**

- **Water and soil contaminated with urine and tissues of infected animals playing a major role in transmitting the disease.**

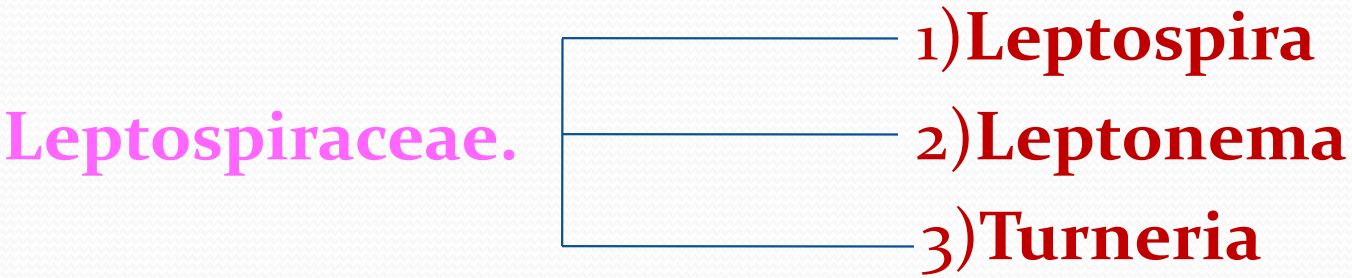
ETIOLOGY

Spirochaete

Order : Spirochaetales

Family:

1. **Leptospiraceae**
2. **spirocheataceae**
3. **Brachyspiraceae**



Spirocheataceae



Genera - 1)Borelia 2)Treponema

Brachyspiraceae - Brachyspira

CLINICAL SIGNS

ANIMALS

- **Anemia**
- **Jaundice**
- **Abortions**
- **Still births, mastitis**
- **Decrease milk production**
- **Death of young calves due to hemolytic anemia and**
- **Reproductive failures.**

Economic impact

- ✓ **Direct or indirect costs of abortions**
- ✓ **Loss of milk production**
- ✓ **Related Vety costs and human infection**
- ✓ **costs associated with vaccination survivellance**
- ✓ **testing of serum samples before international transport.**
- ✓ **Medical treatment**
- ✓ **Loss of productive working time and capacity**

HUMANS

Subclinical infection to severe syndromes of multi organ infection with high mortality.

- ***B*i-phasic fever**
- ***chills , headache & body ache***
- **Severe pain in back, neck and abdomen**
- **Nausea & vomitions**
- **Diarrhea and dry cough**
- **Haemoptysis, Conjunctival suffussion**
- **Hemorrhagic Pneumonitis**
- **Acute respiratory distress**

DIFFICULTY IN DIFFERENTIAL DIAGNOSIS WITH

Animals

- Brucellosis
- IBR
- Listeriosis
- Trichomoniasis
- chronic liver diseases

HUMANS

- Dengue fever
- Hepatitis-B infections
- Influenza infections
- Pulmonary tuberculosis
- Malaria

- **Due to endemicity of the disease in the adjoining states of Tamilnadu, Karnataka and Kerala.**

- **Lack of information on isolation studies in Andhra Pradesh.**

- **A attempt was made for isolation from infected animals, humans and reservoir hosts, rats to find out the epidemiological link between animals, humans and rats.**

- **It also helps to develop suitable vaccine to control the disease.**

- Materials and Methods

Sample collection

Collected during the period from Feb 2006 to May 2010

Clinically suspected cases

Cattle	**- 26**
Sheep	**- 42**
Dogs	**- 13**
Pigs	**- 15**
Humans	**- 53**
Rice feild water	**- 10**
Rats	**- 299**

Table 5: Details of clinical samples collected and leptospiral isolates recovered

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	-	-

Table 4: Details of samples collected from rats for isolation of leptospira

S.No	Place of collection	No.of samples collected	No.of samples tested for isolation	No. of samples positive
1	Govindarajaswamy Temple area	22	22	2 samples positive
2	Railway station area	38	38	3 positive
3	R.S.J unction area	20	20	Negative
4	Bhavani Nagar area	15	15	Negative
5	Bus station area	19	19	Negative
6	Medical college area	185	185	Negative

Preparation of EMJH medium

- **EMJH liquid medium with Tween-80,antibiotics and 5-flurouracil was used as a selective medium.**

- **According to the method of Johnson and Harris (1967) with slight modifications.**

PREPARATION OF MEDIUM

Preparation of leptospira base

- **1.15gms of EMJH base (Difco) in 500ml of Distilled water.**

Preparation of nutrient supplement

- ✓ **Tween 80**
- ✓ **BSA**
- ✓ **Lactalbumin hydrolysate**
- ✓ **5-Flurouracil as a selective agent**

Composition of Stock solutions

Zinc sulphate ($ZnSo_4\ 7H_2O$) (0.4g/100ml)

Calcium chloride ($Cacl_2\ 2H_2O$) (1g /100ml)

Magnesium chloride ($MgCl_26H_2O$) (1g /100ml)

Mangnase sulphate ($MnSO_4\ 4H_2O$) (0.3g/100ml)

Vitamin-B_{12} (0.02g/100ml)

Ferrous sulphate ($FeSO_4\ 2H_2O$) (0.5g/100ml)

Tween 80 (Poly Oxyethylene Mono Olean) (10ml in 90ml of Distilled water)

Composition of working solution

BSA fraction V	5.0 g
$CaCl_2$ (stock solution)	0.5 ml
$ZnSo_4$ (Stock solution)	0.5 ml
$MnSo_4$ (Stock solution)	50.0 µl
$FeSo_4$ (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

- **Base and supplement were sterilized separately and mixed in the ratio of 9:1 and used as a working medium.**

Isolation of leptospira/Leptonema

Processing of samples

Rats : Corticol part of liver and kidneys -10% homogenous suspension – 0.5ml inoculated into 5ml of EMJH medium.

Animals: Bovine, sheep, dogs, humans – blood samples

Pigs-aborted material

- **The clinical samples collected from different species was processed and inoculated into EMJH liquid medium and incubated at 29^0C $\pm 1^0C$ in BOD incubator.**

- **Screened for the presence of the organisms at weekly intervals under Dark Field Microscopy for minimum of 6 weeks.**

PURIFICATION OF LEPTOSPIRA/LEPTONEMA

A) By Filtration

- The isolates obtained from different sources were purified by filtration through 0.22mμ membrane filters and cultured freshly into the EMJH liquid medium.

B) By sub culturing into medium containing 5-flurouracil (100μgs/ml)

C) By Animal Inoculation

By Animal inoculation

- **Pathogenic studies were carried out using guinea pigs (150-250gms).**
- **0.2ml of cultural isolate intraperitoneal inoculation.**
- **From the third day after inoculation, peritoneal fluid is examined under dark field microscopy for the presence of organisms.**

- **0.5ml of blood was collected from heart after 12hrs of post inoculation and inoculated into fresh EMJH liquid medium for re-isolation.**

- **When the inoculated animal dies, liver and kidneys were collected in 10% formalin and processed for Histopathological studies.**

IDENTIFICATION OF LEPTOSPIRA/LEPTONEMA

By dark field microscopy

- **The isolates obtained were initially observed under dark field microscope for their characteristic motility.**

Fontana's staining

- **Done with PD_ADMAS staining kit. Care should be taken to differentiate from fibrils and debris.**

I. PHYSICOCHEMICAL CHARACTERIZATION

A) Growth at 13^0c

- **Isolates were incubated one set at 13^0c and another set at 30^0c along with non pathogenic and saprophytic reference strains.**

- **Growth was checked twice in a week up to 21 days.**

B) Growth in the presence of 8-AZAGUANINE

- ✓ **Incubated the tubes both test and control at 30^0c with and without 8-azaguanine.**

- ✓ **Growth was checked twice in a week up to 21 days.**

C) REACTION ON EGGYOLK AGAR MEDIUM

- ✓ **To study the lipase activity of the isolates.**

D) HISTOPATHOLOGY

- ✓ **Liver and kidneys from the inoculated guinea pigs were collected in 10% formalin for histopathological studies and stained with haematoxylin and eosin for examination.**

II. MOLECULAR CHARACTERIZATION

16SrRNA PCR

- **DNA was extracted from cultural isolates according to the method of Boom et al, 1990 by CTAB method.**
- **16SrRNA PCR was carried out according to the method of Shukla et al, 2003 with certain modifications.**
- **PCR was carried out with a set of primers 1 and 2.**

PRIMERS-16srRNA PCR

S.No	Primer used	Sequence
1	F	5’ GGC GGC GCG TCT TAA ACA TG 3’
2	R	GTC CGC CTA CGC ACC CTT TAC G 3’

CYCLIC CONDITIONS

Initial Denaturation at 94^0C – 5min

↓

Annealing at 63^0C – 45sec

↓

Extension at 72^0C – 60sec

Followed by cyclic denaturation at 94^0C – 1min

50µL of reaction mixture consisting of

MgCl2 (25mM)	-	3.0µL
dntps (200mM)	-	1.0µL
Tris HCL (10mM)	-	5.0µL
KCL (50mM)	-	2.0µL
taq DNA polymerase (0.5u)		2.0µL
primers (20Pmol)		
forward	-	1.0µL
reverse	-	1.0µL
template DNA	-	2µL
DEPC water	-	33µL

- **Amplified products were analyzed with 1% agarose gel and stained with Ethidium Bromide.**

- **The amplified product is visualized under U.V transilluminator or gel documentation.**

- **The expected amplified product size is 525bp with 1kb DNA ladder.**

DNA SEQUENCING ANALYSIS

- **Amplified PCR product of 16SrRNA was sent to M/s. Bio serve technologies private limited, Hyderabad for sequencing.**

- **The results of sequence was subjected to nucleotide sequence analysis and homology was studied using BLAST N search (Clustal W2)**

RESULTS AND DISCUSSION

- **Leptospira can be diagnosed by several laboratory methods of which serological methods are widely used.**
- **But isolation allows definite diagnosis of individual infection.**
- **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 from naturally infected animals and from reservior hosts like rodents is available.**

- **Information available on leptospirosis is limited to serology.**

- **Hence, in the present work attempt was made for isolation and characterization.**

DETAILS OF ISOLATES RECOVERED

- **A total of 17 isolates were recovered from different species upon culturing from 458 suspected field samples with percent positivity of 3.70.**

Sheep	**-**	**42-5**
Pigs	**–**	**15-4**
Humans	**-**	**53-2**
Rice field water	**-**	**10-1**
Rodents	**-**	**299-5**

- **During the study leptospiral organisms were isolated in EMJH liquid medium and maintained in EMJH semi solid medium.**

- **Earlier workers Ellis et al, (1981); Gregore et al, (1987); Faine et al, (1991); Leonard et al,(1992); Gangadhar Rao et al, (2000) and Vijayachari et al (2003) were used the same medium for isolation and maintenance in their studies.**

- **Shown Berg (1981) reported the harmful effects of the antibiotics on leptospiral growth after 48hrs of incubation.**

- **However, such effect of antibiotics on growth of the organisms was not noticed during the present study.**

- **During the study leptospiral organisms were observed in EMJH medium between 12-14 weeks of inoculation.**

- **Vijayachari et al (2007) and Gibson et al (2008) were also observed leptospira between 12-14 weeks.**

- **Keeping in view of slow growth of organisms the WHO (2003) suggested observing period for 4-6 months for isolation of leptospira.**

- **Isolation 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 from clinical samples is an art require skill and experience.**

Successful isolation depends

- ✓ **Time of collection**
- ✓ **Interval between collection and processing**
- ✓ **Number of organisms present in the clinical samples**
- ✓ **Interference of contaminating organisms**
- ✓ **Tissue autolysis (Theirmann 1984, Venkatesha 2006 and OIE 2008**

PURIFICATION

- **All the isolate recovered during the period were purified by**

1) 0.22mμ membrane filters

2) Sub culturing with selective agent

5-flurouracil

3)Animal inoculation:

- ✓ **All the seventeen isolates recovered on cultural isolation inoculated in 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.**
- ✓ **Were maintained in semisolid medium for further studies**

IDENTIFICATION

DARK FIELD MICROSCOPY

- **The characteristic motility of flexion, extension and rotational movements were observed suggestive of leptospira.**

- **Where as two of the isolates (S-1 and RG-1) were found to have sluggish motility which is quite different from that of leptospira and were suggestive of leptonema**

FONTANA'S STAINING

- **Seventeen(17) isolates recovered in pure form upon staining observed the characteristic morphology of spiral shape with hooked ends.**

- **Appeared as brownish black with yellow color background.**

- **Gangadhar and Rajasekhar, 1998 also used the same method and reported the presence of the organisms**.

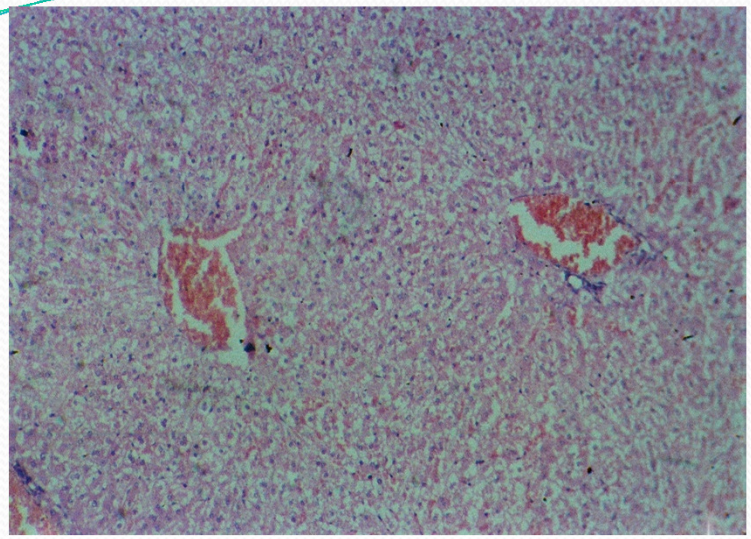
HISTO PATHOLOGY

- **Vasculitis, endothelial damage, infilteration of monocytic cells, plasma cells, Histiocytes and esoinophils.**
- **On gross examination – Petical Haemorraghes**
  Organ are often discolored due to icterus
- **Histopathology – More marked in liver, Kidneys, heart and lungs**

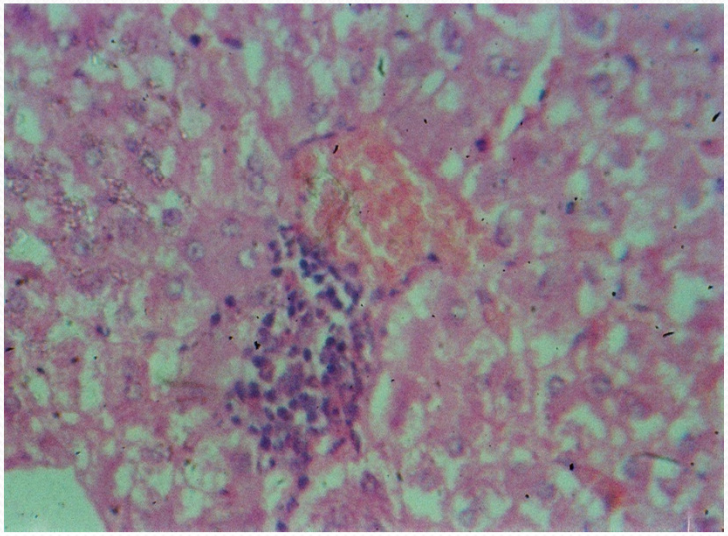
✓ **Liver – Hypertrophy,hyperplasia and erythrophagocytosis**

✓ **Kidney – interstitial nephritis, intense cellular infiltration of neutrophils and monocytes Presence of leptospires in renal tubules**

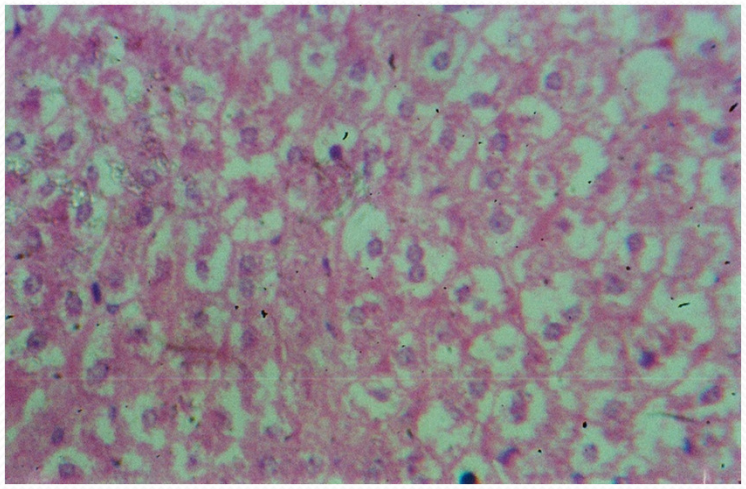
Histo-pathology in Liver



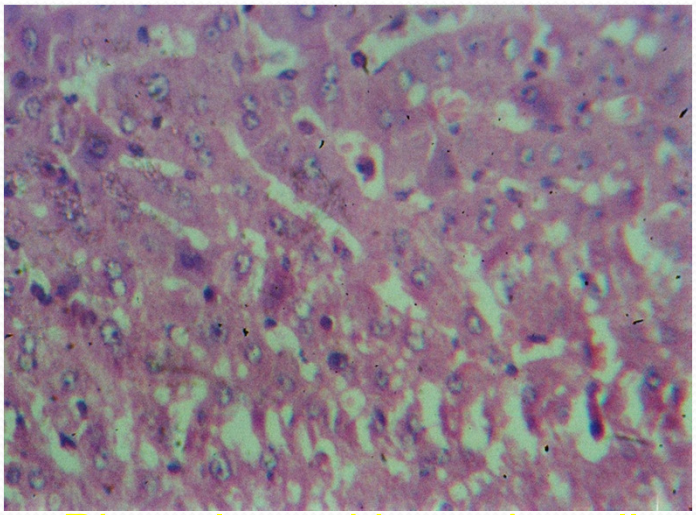
Severe Congestion



Perivascular mononuclear

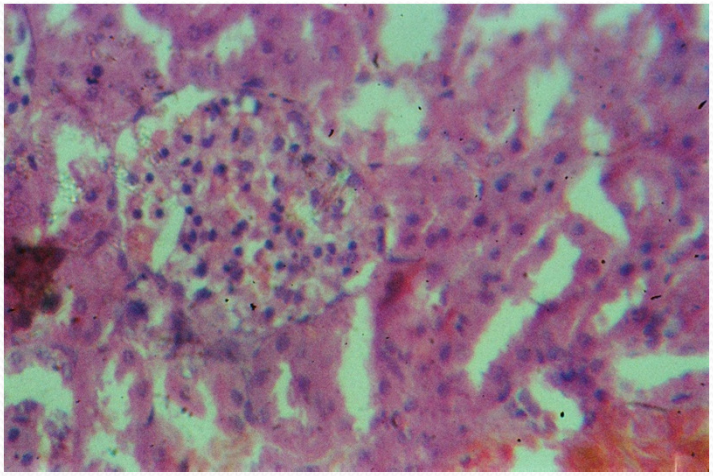


Hepatic vacuolation

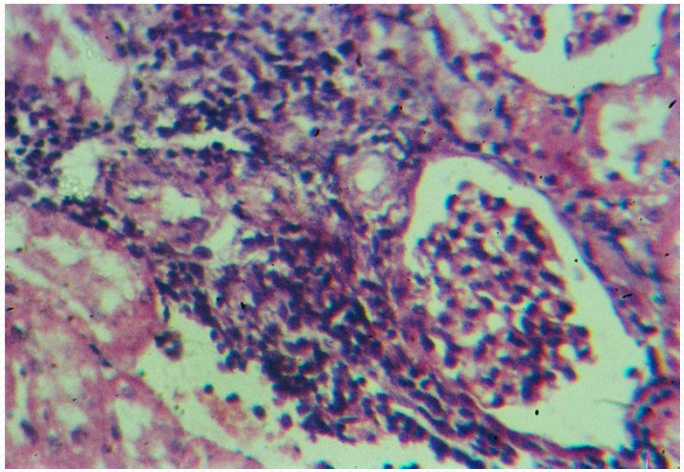


Bi-nucleated hepatic cells

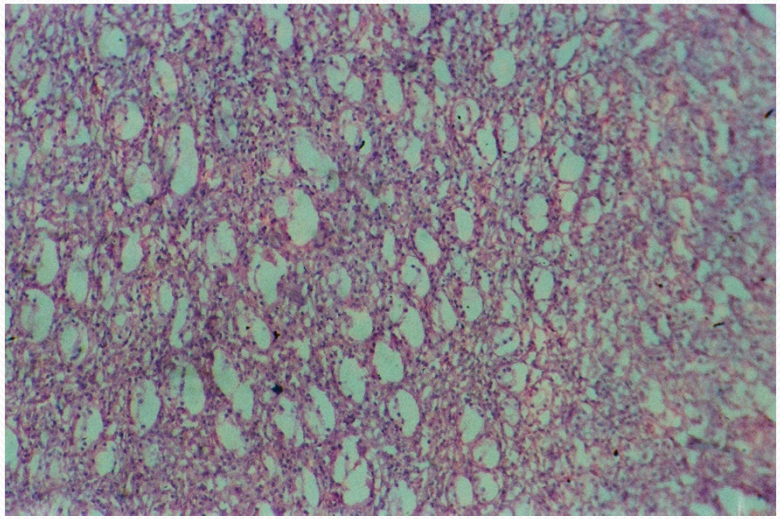
Histo-pathology in Kidney



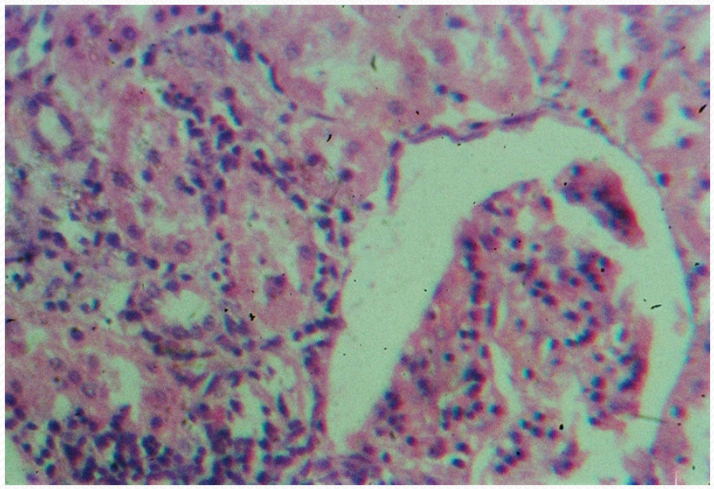
Globulular infiltration



Inter tubular infiltration



Cystic dilatation of tubules



Intra tubular infiltration of mononuclear cells

PHYSICO CHEMICAL CHARACTERIZATION

- **Characterization of isolates is an important tool for identification.**

- **Isolates were confirmed conventionally using physicochemical characterization and was assessed by studying the growth at 13^0C and 8-azaguanine test and reaction on egg yolk agar.**

- **To differentiate pathogenic leptospira from non-pathogenic leptospira.**

GROWTH AT 13^0C

All the seventeen isolates failed to grow at 13^0c indicating pathogenecity.

Table 25: 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	-	-	-	-	+	+	-
7.	Dog	13	-	-	-	-	+	+	-

8-AZAGUANINE TEST

All the seventeen isolates did not grown in the presence of 8-azaguanine indicating pathogenecity.

Table 26: 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 seventeen isolates showed lipase activity on egg yolk agar medium indicating virulence.



Lipase activity of leptonema (S-1 isolate) on egg yolk agar

- **Johnson and Rogers,1964**
- **Johnson and Harris,1967**
- **Noubade et al,2002**
- **Vijayachari et al, 2003**
- **Shukla et al, 2003 - also used same characterization method in differentiating pathogenic organisms.**

MOLECULAR CHARACTERIZATION

- **All the seventeen isolates were subjected for 16SrRNA PCR for specific identification of organisms by amplification of 16SrRNA conserved gene using specific primers 1 and 2.**
- **The primers 1 and 2 used in the study amplified the expected gene and yielded 525bp.**
- **The analysis of sequence results of 16SrRNA PCR showed that**
  - ✓ **One of the sheep isolate (s-1) showed 100 percent homology with *leptonema ilini.***
  - ✓ **One of the other isolate from rat (RG-1) showed 98 percent homology with leptonema ilini.**

- **Hence 16SrRNA PCR is simple and specific method of identification of pathogenic leptospira/leptonema from different species.**

- **Several workers used 16SrRNA PCR for identification of pathogenic leptospires.**

Merien et al, 1992

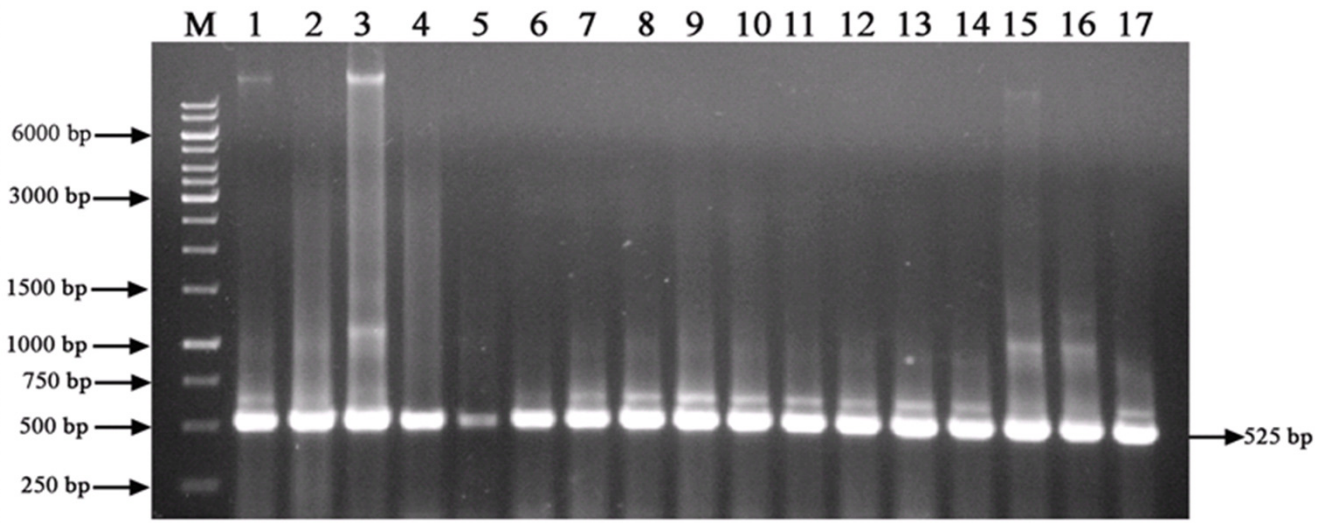
Shukla et al, 2003

Turk et al, 2003

Wangroongsarb,2005

Djadid et al, 009

Fig.16: 16S rRNA – PCR for leptospiral isolates



M : Molecular weight marker 250 bp

1 : S1 isolate

2 : S2 isolate

3 : S3 isolate

4 : RR2 isolate

5 : RG2 isolate

6 : S4 isolate

7 : S5 isolate

8 : RR1 isolate

9 : RR3 isolate

10 : RG1 isolate

11 : H1 isolate

12 : W1 isolate

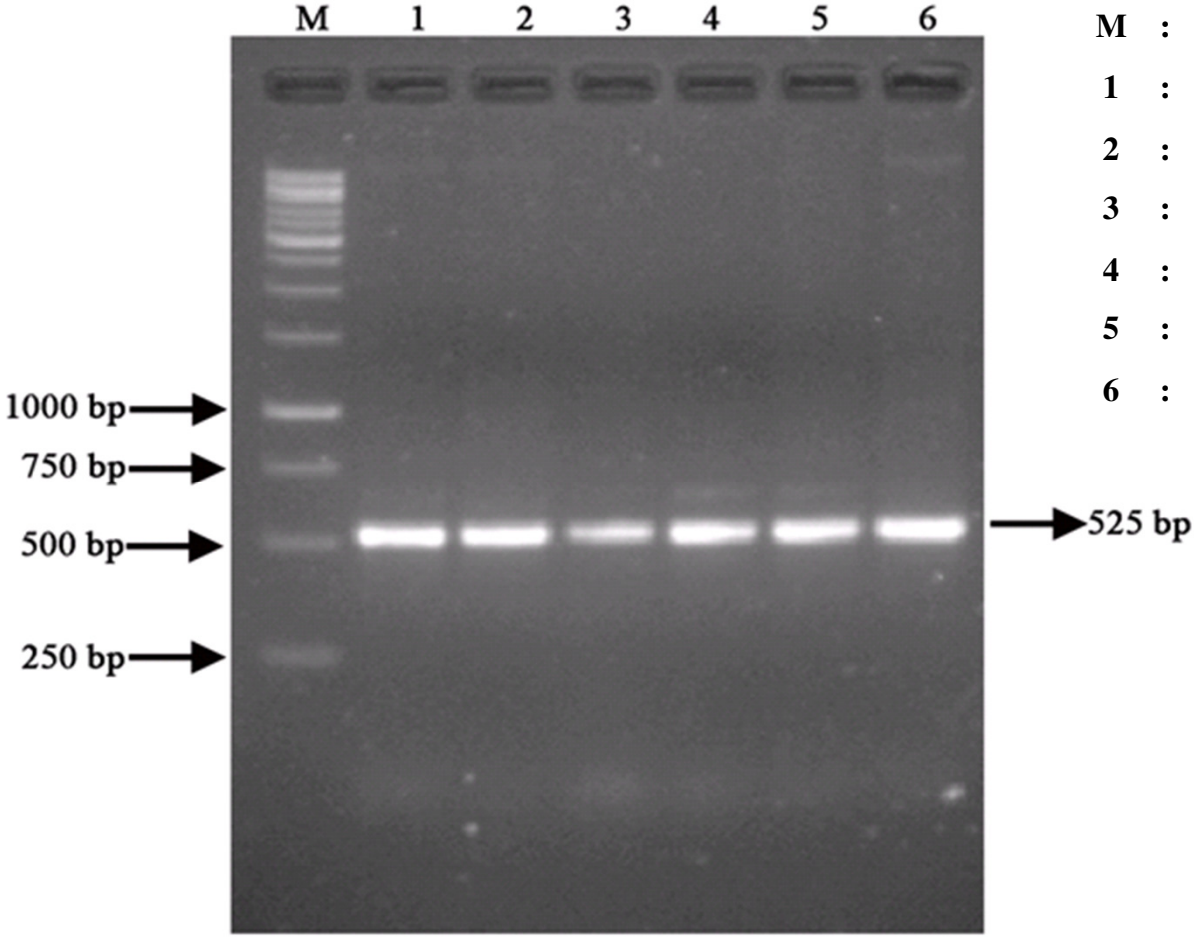
13 : P1 isolate

14 : P2 isolate

15 : P3 isolate

16 : P4 isolate

Fig.17: 16S rRNA – PCR for leptospiral isolates sent for sequencing



M : Molecular weight marker 250 bp

1 : Sample 1 (S1 isolate)

2 : Sample 3 (S3 isolate)

3 : Sample 6 (S4 isolate)

4 : Sample 8 (RR1 isolate)

5 : Sample 10 (RG1 isolate)

6 : Sample 16 (P4 isolate)

SEQUENCE –RESULTS

Sl.No	Isolate	16SrRNA
1.	S-1	*Leptonema ilini*
2.	S-3	*l.Hardjo*
3.	S-4	*L.Inadi*
4.	RR-1	*L.Noguchi*
5.	RG-1	*Leptonema.Ilini*
6.	P-4	*L.pomona*

- **First isolation and characterization of leptospiral isolates - Andhra Pradesh.**
- **Apart from *L.hardjo* and *L.pomona*, rare serovars *L.noguchi and L.inadai* were also isolated and identified.**
- **Though leptonema was considered as non-pathogenic strain**

  Teresa et al,1993,

  Hlavatz and Bozobska ,1994

  Gangadhar et al, 2005

Reported the pathogenic role of leptonema.

Further detailed investigations are required on the isolates

L.illini L.noguchi and L.inadai

Leptonema

- **Spirochete closely related to leptospira but classified under separate genus.**
- **Morphologically similar to leptospires but ultra structurally different .**
- **Presence of cytoplasmic tubules –**
  - ***a pair of basal discs***
  - ***ability to grow in tryptic soya broth***
- **Saprophytic in nature, however there are stray reports indicating pathogenecity (Hlavatz and Bazubska, 1974 and Teresa et al., 1993).**

- **Gangadhar et al., 2005 reported the isolation of leptonema from rodents and rabbits with a history of repeated abortions.**

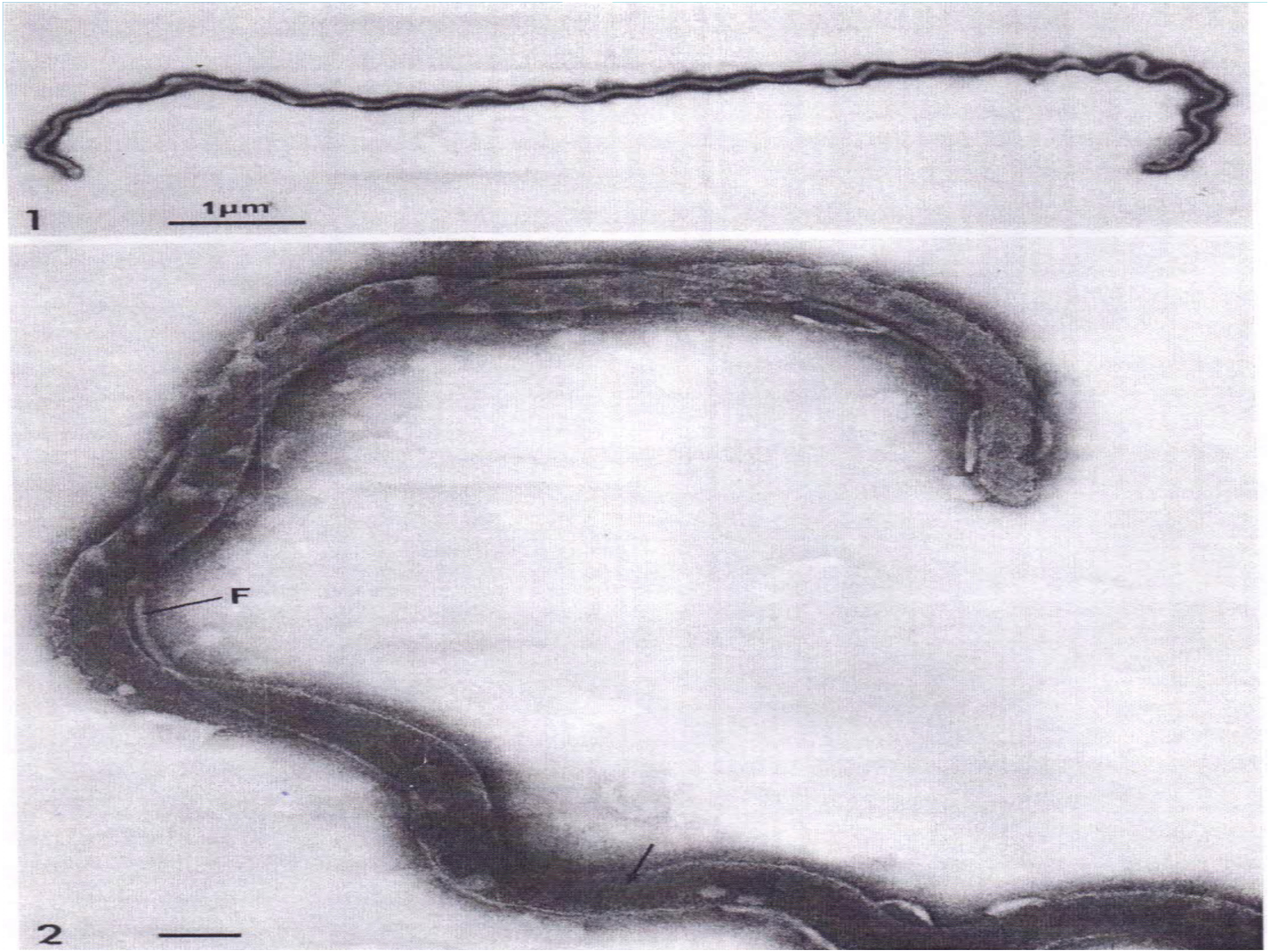
- **There are reports of isolation from sheep and rodents.**

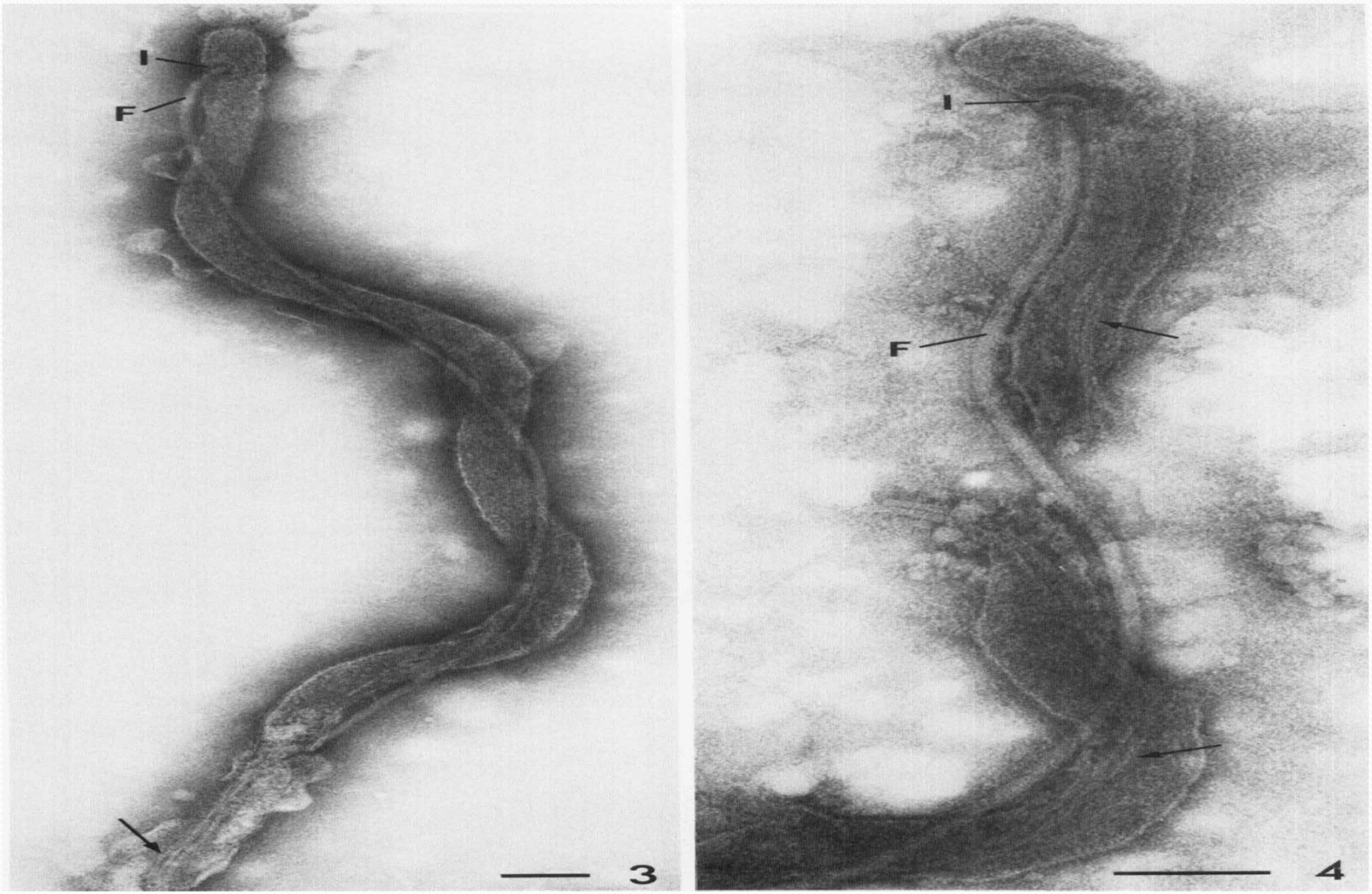
- **G+C content of leptonema differs from other leptospires**

  53 percent moles – l.ilini strain 3055

  51.2percent moles –l.ilini strain 177

But in leptospira G+C varies from 35.2 to 41.2 percent moles


1
1µm
F
2


I
F
3
I
F
4

CONCLUSION

- **A total of 159 samples collected from different species cattle, sheep, pigs, dogs, humans and rats were processed for isolation studies.**
- **EMJH liquid medium is used as a selective medium for isolation and EMJH semi solid medium for maintenance.**
- **A total of seventeen isolates were recovered sheep(5), pigs(4), rats(5), humans(2) and rice field water(1).**
- **All the isolates were purified.**

- **All the isolates were subjected for physico chemico characterization to differentiate pathogenic from non-pathogenic.**

- **All the isolates were subjected for molecular characterization.**

- **Andhra Pradesh having tropical climate, receiving rains from south west monsoons, involved in Agricultural, animal Husbandry and fishing activities provides suitable climatic conditions for the survival of leptospires/leptonema.**

- **However, detailed investigations are required for mapping of serovars prevailing in the state to select candidate vaccine strains for proper**

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Thank You