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RESEARCH ARTICLE

OCCURENCE OF LEPTONEMA FROM ANDHRA PRADESH

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ABSTRACT

During the process of isolation and identification of leptospira from different parts of Andhra Pradesh Leptonema organisms were also isolated and identified. A total of 458 clinically suspected samples were collected from suspected cases of cattle (26), sheep (42), dogs (13), pigs (15), humans (53), rats (299) and stagnant water from rice field (10) and subjected to isolation of leptospira using EMJH liquid medium. A total of seventeen (17) isolates were recovered from sheep (5), rats (5), pigs (4), humans (2) and rice field water (1) on isolation. Out of 17 isolates two isolates were found to have sluggish motility which is different from that of leptospira and suggestive of leptonema.

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INTRODUCTION

Leptospirosis is a wide spread zoonotic disease (Turner, 1970). Affecting humans and wide variety of animals (Vinetz *et al*., 2001). 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 and 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. Due to endemicity of the disease in the adjoining states of Tamilnadu, Karnataka and Kerala. Lack of information on isolation studies in Andhra Pradesh. An attempt was made for isolation from infected animals, humans, reservoir hosts and rats to find out the epidemiological link between animals, humans and rats. It also helps to develop suitable vaccine to control the disease.

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MATERIALS AND METHODS

Sample collection

Collected during the period from Feb 2006 to May 2010 from clinically suspected cases Cattle, Sheep, Dogs, Pigs, Humans, Rice field water and Rats (Table 1)

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 consisting of Tween 80, BSA, Lact albumin hydrolysate,5-Flurouracil as a selective agent.

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 Leptonema

Processing of samples

Rats

10% homogenous suspension was made from cortical part of liver and kidneys. From this 0.5ml were inoculated into 5ml of fresh EMJH medium.

Animals and humans: Blood samples

Pigs: Aborted material

Rice field water: Human outbreak area

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 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: 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 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.

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 egg yolk agar medium: To study the lipase activity of the isolates.

Molecular characterization

a) 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.

B) *Primers for16SrRNA 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, followed by Annealing at 63^0C – 45sec, Extension at 72^0C – 60sec and followed by cyclic denaturation at 94^0C – 1min for 32 cycles. 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.

Sequence analysis: Amplified PCR product of 16SrRNA was sent to M/s. Bio serve technologies private limited, Hyderabad for sequencing. The results of sequence were subjected to nucleotide sequence analysis and homology was studied using BLAST N search (Clustal W2).

RESULTS

A total of seventeen isolates were recovered from different species upon culturing from 458 suspected clinical samples with percent positivity of 3.7 (Table 1).

Purification

All the isolate recovered during the period were purified by 0.22mµ membrane filters, sub culturing with selective agent i.e.5-flurouracil and by 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.

Table 1. Details of clinical samples collected and 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	-	-
TOTAL		458	17	3.71

Table 2. Growth characterization of leptospiral isolates

S.No.	No. of samples tested	No. of leptospiral Isolates obtained		Growth at		Controls			
						Reference pathogenic		Reference Non -pathogenic	
				13^{O}C	30^{O}C	13^{O}C	30^{O}C	13^{O}C	30^{O}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	-	-	-	-	+	+	-

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						

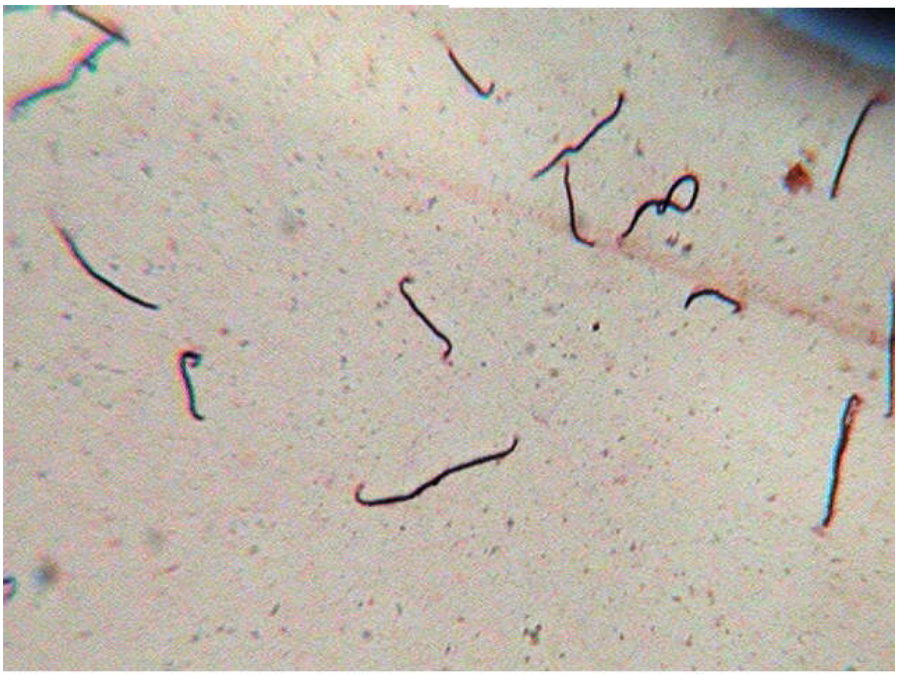

Fig. 1. Fontana's staining of Leptonema – S1

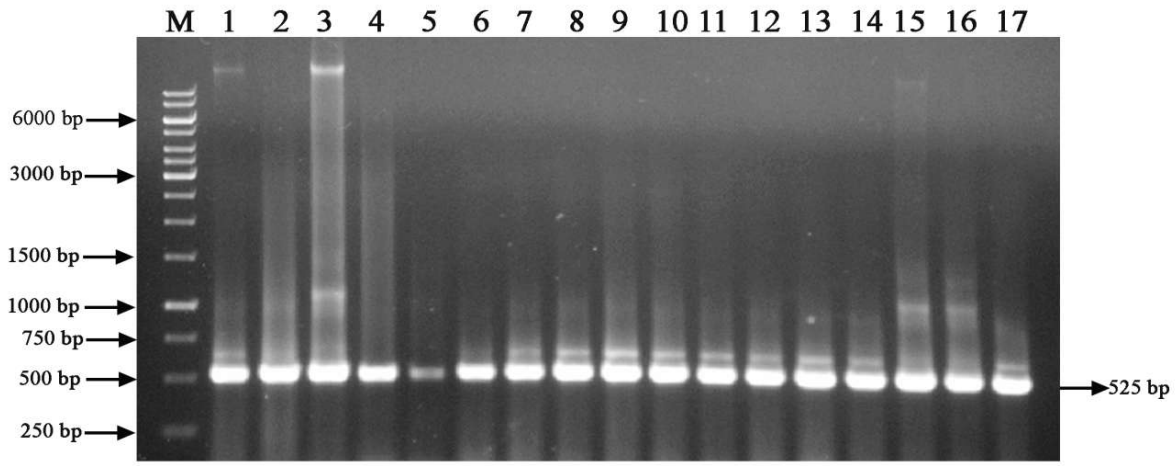

Fig.2. 16s rRNA – PCR for leptospiral isolates

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

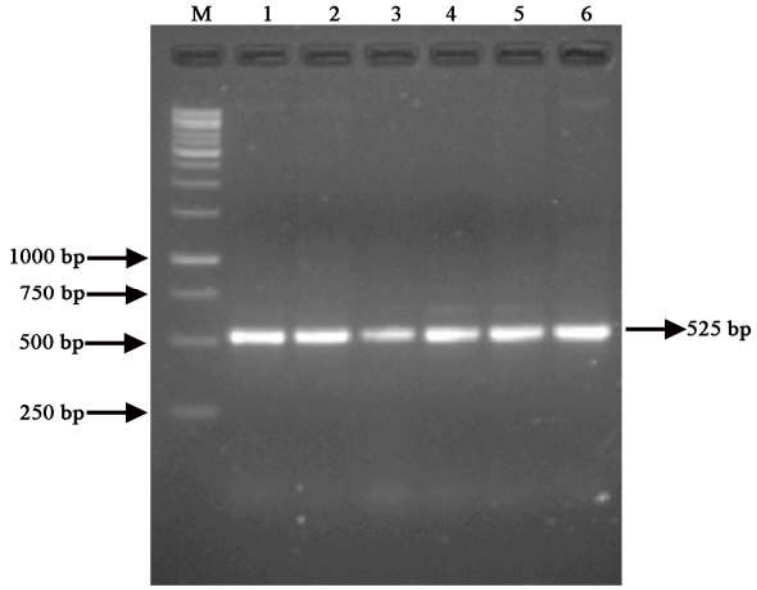

Fig.3. 16s rRNA – PCR for leptospiral isolates sent for sequencing

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)

All the seventeen isolates 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. Whereas 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. (Fig.1)

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 pathogenicity. (Table 2)

8-azaguanine test

All the seventeen isolates did not grown in the presence of 8-azaguanine indicating pathogenicity. (Table 3)

Reaction on egg yolk agar

All the seventeen isolates showed lipase activity on egg yolk agar medium indicating virulence.

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. Fig 2 The analysis of sequence results of 16SrRNA PCR Fig.3 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.

DISCUSSION

First isolation and characterization of Leptonema in Andhra Pradesh. Though several methods are there for diagnosis of any pathogen but isolation allows definite diagnosis of individual infections. It also helps to study the 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 resources host like rodents in available. Hence, the present work was attempted for isolation and characterization of leptonema. Earlier Hanson *et al*, 1974, Kari Hovind hougen, 1979 reported that trypticase soya broth and long chain fatty acids are required for the growth of leptonema. But surprisingly during our study the leptonema isolates wee adopted to grow in EMJH liquid medium and maintained in semi solid agar medium along with leptospira isolates. On fontana's staining the leptonema morphology is similar to leptospira, spiral shape with hooked ends and appeared as brownish black with yellow black ground. Similar observation was also made by Bazovska *et al*. (1983). Johnson and Harris, 1967 reported that *Dimbovitza* strain of leptonema grow at 13^0C and Johnson and Rogers (1964) studied the growth of the leptonema in the presence of 8-azaguaine. But during over study the two isolates of leptonema did not grown neither at 13^0C nor in the presence of 8-azaguanine and also lipase activity on egg yolk agar medium indicating the pathogenicity. On molecular characterization, two of the isolates sheep (S-1) and rat (RG-1) were showed 100% and 98% homology with Leptonema illini. Further the S-1 isolate recovered from sheep having symptoms of pyrexia, anemia and haemoglobinuria suggestive of pathogenic role of leptonema. Earlier Halvatz and Bazubsk 1974 and Teresa *et al* 1993 also stated the pathogenecity of the strain Dimbovitza belonging to the genus Leptonema. Gangadhar *et al* 2005 repoted the isolation of Leptonema from rodents and rabbits with the history of repeated abortions. Hence 16SrRNA PCR is considered to be simple and specific method for the identification of Leptonema.

Conclusion

A total of 458 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. All the isolates were purified using 0.22µ membrane filters and animal inoculation. A total of seventeen isolates were recovered sheep (5), pigs (4), rats(5), humans(2) and rice field water(1). All the isolates were subjected for physico chemico characterization to differentiate pathogenic from non-pathogenic. All the isolates were subjected for molecular characterization using 16SrRNA PCR with specific primers. The results of sequence of DNA analysis revealed the presence of Leptonema. Of the two isolates of Leptonema, one from sheep and another from rat were recovered and shown 100 percent and 98 percent homology with leptonema illini.

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